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(54) Title: MPL LIGAND ANALOGS

(57) Abstract

Mpl ligand analogs having one or more changed glycosylation sites as compared to a naturally occurring mpl ligand sequence of a corresponding number of amino acids are disclosed. The invention also relates to DNA sequences encoding said mpl ligand analogs, recombinant plasmids and host cells for analog expression, and therapeutic compositions including such analogs.

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# MPL LIGAND ANALOGS

## Field of the Invention

The present invention relates to mpl ligand analogs having at least one changed O- or N-linked glycosylation site. The invention also relates to DNA sequences encoding these mpl ligand analogs, and recombinant plasmids and host cells for analog expression.

## Background of the Invention

MGDF, or megakaryocyte growth and development factor, is a recently cloned cytokine that appears to be the major regulator of circulating platelet levels. See Bartley, T.D. et al., *Cell* **77**:1117-1124 (1994); Lok, S. et al., *Nature* **369**:565-568 (1994); de Sauvage, F.J. et al., *Nature* **369**:533-538 (1994); Miyazake, H. et al., *Exp. Hematol.* **22**:838 (1994); and Kuter, D.J. et al., *PNAS USA*, **91**:11104-11108 (1994). MGDF as described in Bartley, T.D. et al., *Cell* **77**:1117-1124 (1994), is also referred to as thrombopoietin (TPO), mpl ligand, and megapoietin. Herein, the term "mpl ligand" will be used generically to refer to all polypeptides that activate the mpl receptor, including TPO and MGDF. The mpl receptor is a cell surface protein that, upon activation, leads to production and/or development of megakaryocytes and platelets. See WO 92/07074.

"Mpl ligand analogs" are polypeptides that differ from native sequences in a way that affects the number, location or type of carbohydrate linkage sites. Such polypeptides are one aspect of the present invention. Mature native human mpl ligand is a protein

having 332 amino acids in total. The sequence of this protein (attached to a 21-amino acid long leader sequence) and the corresponding cDNA are shown in FIG. 1 herein (SEQ. ID NOs.: 1 and 2).

Recombinant mpl ligand produced in both Chinese Hamster Ovary (CHO) and *E. coli* cells has been demonstrated to have a biological activity of specifically stimulating or increasing megakaryocytes and/or platelets *in vivo* in mice, rats and monkeys. See e.g., Hunt, P. et al., *Blood* 84(10):390A (1994). Human mpl ligand molecules that have been truncated (as compared to the 332 amino acid protein encoded by the cDNA in humans) so that they extend at least 151 amino acids, starting from amino acid position 22 in FIG. 1, retain biological activity *in vivo*. FIG. 2 (SEQ. ID NOs.: 3 and 4) shows one example of a truncated mpl ligand molecule which, in mature form, has 174 amino acids and has biological activity. In FIG. 2, the 174 amino acid long protein is shown attached to a 21 amino acid N-terminal leader sequence. This molecule was used to create some of the mpl ligand analogs in the examples section below. Other analogs are based on amino acids 1-199, 1-191, and 1-183 of FIG. 1. It is also possible to remove up to the first six amino acids at the N-terminus of the mature human sequence mpl ligand protein and retain biological activity. Therefore, it appears that biological activity is retained within amino acids 7 to 151 (inclusive) of the mature amino acid sequence of human mpl ligand shown in FIG. 1.

In general, many cell surface and secretory proteins produced by eukaryotic cells are modified with one or more oligosaccharide groups. This modification, referred to as glycosylation, can dramatically affect the physical properties of proteins and can also be important in protein stability, secretion, and

subcellular localization. Proper glycosylation can be essential for biological activity. In fact, some genes from eukaryotic organisms, when expressed in bacteria (e.g., *E. coli*) which lack cellular processes for glycosylating proteins, yield proteins that are recovered with little or no activity by virtue of their lack of glycosylation.

Glycosylation occurs at specific locations or sites along the polypeptide backbone and is usually of two types: O-linked oligosaccharides are attached to serine (Ser) or threonine (Thr) residues while N-linked oligosaccharides (chains) are attached to asparagine (Asn) residues when they are part of the sequence Asn-X-Ser/Thr, where X can be any amino acid except proline. X is preferably one of the 19 naturally occurring amino acids not counting proline. The structures of N-linked and O-linked oligosaccharides and the sugar residues found in each type are different. One type of sugar that is commonly found on both is N-acetylneuraminic acid (hereafter referred to as sialic acid). Sialic acid is usually the terminal residue of both N-linked and O-linked oligosaccharides and, by virtue of its negative charge, may confer acidic properties to the glycoprotein.

As used herein glycosylation "sites" are amino acid residues that are structurally able to link to glycosyl residues, although such sites may or may not be actually linked to a glycosyl residue. As noted above, O-linked sites are either Ser or Thr residues, whereas N-linked sites are either Asn-X-Ser or Asn-X-Thr, where X is defined as any amino acid other than Pro (preferably one of the 19 naturally-occurring amino acids, excluding Pro). Whether a given site is glycosylated with a glycosyl chain is determined by the host cell in which the molecule is expressed, the amino acids neighboring the site, and other factors.

As used herein, the number of "chains" attached to a given mpl ligand analog will be the average number of carbohydrate (i.e., glycosyl) chains attached to a given mpl ligand molecule expressed by a particular host cell. Notably, the glycosylation sites for natural and corresponding recombinant mpl ligand will generally be the same, whereas the number of chains will possibly vary depending upon whether the particular host cell used for recombinant expression attaches glycosyl chains to the same sites or not, as compared to the natural source. Herein, whenever a comparison is made between recombinant and natural mpl ligand analogs, the same number of amino acids will be compared, regardless of whether the natural source actually produces an mpl ligand molecule having that length. Thus, "natural" refers to the sequence employed in a particular species (such as human) rather than the length of the molecule actually expressed in such natural source.

Naturally occurring mpl ligand is a glycosylated molecule. The glycosylation pattern of natural mpl ligand is related to two key domains that have been found in mpl ligand. The sequence of the first approximately 151 amino acids of mature human mpl ligand, corresponding to an active portion of the molecule, bears notable homology to erythropoietin (EPO), a cytokine capable of stimulating production of erythrocytes, and is referred to as the "EPO-like" domain of human mpl ligand. The remaining amino acids of the mature protein make up a so-called "N-linked carbohydrate" domain, since they include most if not all of the natural sites for N-linked glycosylation. In human mpl ligand, there are six N-linked glycosylation sites all contained in the N-linked glycosylation domain. Both domains contain O-linked glycosylation sites. There are an estimated 12-14 O-linked

glycosylation chains in the molecule. Experimental evidence with human mpl ligand DNA expressed recombinantly in CHO cells reveals that in the EPO-like domain at least two O-linked sites are glycosylated, at positions 1 (Ser) and 37 (Thr).

Glycoproteins such as mpl ligand can be separated into different charged forms using techniques such as isoelectric focusing (IEF). For example, several parties have reported IEF studies of crude and partially purified erythropoietin preparations (Lukowsky et al., *J. Biochem.* **50**:909 (1972); Shelton et al., *Biochem. Med.* **12**:45 (1975); Fuhr et al., *Biochem. Biophys. Res. Comm.* **98**:930 (1981)).

In spite of the above information on glycosylation of mpl ligand molecules, there remains a need to obtain mpl ligand molecules having a different glycosylation pattern and which retain or have improved biological activity.

Accordingly, it is an object of the present invention to provide novel glycosylated mpl ligand molecules, referred to as mpl ligand analogs. It is a further object of this invention to provide pharmaceutical compositions containing such molecules and methods of treating conditions treatable by mpl ligand with the mpl ligand analogs of this invention.

## Summary of the Invention

In one embodiment, the subject invention relates to analogs of mpl ligand comprising an amino acid sequence which includes at least one added, at least one deleted, and/or a combination of at least one added and deleted, site for glycosylation as compared to the corresponding natural sequence mpl ligand. The added or deleted site(s) for glycosylation may result in a greater or lesser number of carbohydrate chains, and higher or lower sialic acid content, than corresponding

natural sequence mpl ligand, particularly human mpl ligand. For example, one type of analog could involve deleting one or more N- or O-linked sites, and addition of one or more N- or O-linked sites at the same or another position.

In another aspect of the above embodiment, the subject invention relates to mpl ligand analogs comprising amino acid sequences which involve replacement of one or more N- or O-linked glycosylation sites with one or more non-naturally occurring sites. Thus, an N-linked site may be replaced with a different N-linked site; an N-linked site may be replaced with an O-linked site; an O-linked site may be replaced with a different O-linked site; and an O-linked site may be replaced with an N-linked site.

Combinations of any of the above changes are further encompassed within this invention.

The invention further encompasses DNA sequences encoding such mpl ligand analogs, and recombinant plasmids and host cells for analog expression.

In all of the above cases, the change in glycosylation site results in a change in the number, amount, location or type (N- vs. O-) of glycosyl chains in the resulting mpl ligand analog and retains a biological activity of mpl ligand, i.e., the analog can still activate the mpl receptor. Activation of the mpl receptor means that megakaryocytopoiesis is enhanced thereby resulting in an increase in platelets *in vivo*.

## Brief Description of the Drawings

FIG. 1 shows the DNA and amino acid sequence of native human mpl ligand including a signal peptide (amino acids -21 to -1) and the mature amino acid sequence (1-332).

FIG. 2 shows the DNA and amino acid sequence of mpl ligand corresponding to amino acids 1-174 of the

human mature mpl ligand sequence attached to a 21 amino acid long signal peptide. The sequences flanking the coding regions have introduced XbaI and SalI cloning sites at the 5' and 3' ends respectively.

FIG. 3 shows a Western blot with *E. coli* and CHO expressed mpl ligand. MK stands for Met-Lys, which is added to the N-terminus of mpl ligand for expression in *E. coli*, and may be cleaved off using a dipeptidase, such as cathepsin C. A molecule in which MK has been removed is referred to as desMK. Treatment with the glycosidases neuraminidase and O-glycanase is indicated.

FIG. 4 shows *in vivo* activity of *E. coli* and CHO expressed mpl ligand in normal mice, in terms of platelet counts. The data indicates that glycosylated mpl ligand (CHO material) has superior activity than non-glycosylated (*E. coli*) material. This may be a result of increased half-life for the glycosylated material. For example, CHO 332 stands for human mpl ligand amino acids 1-332 (FIG. 1) expressed in CHO cells.

FIG. 5 shows a Western blot analysis of COS cell supernatants of recombinant human mpl ligand and analogs 4, 6, 7, 9, 10, and 11. The construction of the analogs is described in Example 4. Analogs 4, 7, 10 have at least one additional carbohydrate chain as evidenced by slower gel mobility. The analog numbers correspond to analog numbers provided in Table 1 (e.g., 11 corresponds to analog N11). The control is N1 in Table 1.

FIG. 6 shows a Western blot analysis of COS cell supernatants of recombinant human mpl ligand and analogs 4, 5, 13, 14, and 15. The construction of the analogs is described in Example 4. Analogs 4, 13, 14, and 15 have at least one additional carbohydrate chain as evidenced by slower gel mobility.

FIG. 7 shows a Western blot analysis of COS

cell supernatants of human mpl ligand and indicated mpl ligand analogs after treatment with N-glycanase. The results indicate that the analogs have differential glycosylation patterns.

FIG. 8 shows the results of a human megakaryocyte growth bioassay with mpl ligand analogs. Panels A and D are the positive and negative controls respectively. The well pictured in panel A received 37.5 pg of wild type (i.e., natural sequence) mpl ligand 1-174 COS-1 conditioned medium and shows substantial megakaryocyte growth. Panel D received 1.5 ul of COS-1 mock conditioned medium and shows no growth. Panels B and C are mpl ligand 1-174 analogs 7 and 10 respectively. Panel B received 9.0 pg of mpl ligand COS-1 conditioned medium while panel C received 27 pg and both show excellent megakaryocyte growth.

FIG. 9 shows a Western blot analysis of CHO mpl ligand 1-174 and analogs N4 and N15 (see Table 1). Slower gel mobility demonstrates that analog N4 (4B) has one additional oligosaccharide while analog N15 (15-8) has two additional oligosaccharides.

FIG. 10 shows a Western blot of CHO cell-produced mpl ligand analogs with and without treatment with N-glycanase as indicated. Slower gel mobility after treatment with N-glycanase demonstrates the presence of N-linked oligosaccharide.

FIG. 11 shows platelet counts from mice treated with various forms of mpl ligand at various doses. The data demonstrate that increased amounts of N- and/or O-linked carbohydrate result in increased *in vivo* activity.

FIG. 12 shows a Western blot analysis of COS-produced mpl ligand 1-174, along with analogs N10, N15, N33, N39, N31, N35, and N40. The number of added N-linked glycosyl sites is also indicated. The figure shows that increasing the number of N-linked sites

reduces the mobility of mpl ligand due to increasing amounts of N-linked carbohydrate.

FIG. 13 shows a Western blot analysis of COS-produced mpl ligand 1-174, along with analogs N15, N29, N30, and N38. The number of N-linked glycosyl chains is also indicated.

## Detailed Description of the Invention

The subject invention provides mpl ligands with different glycosylation sites as compared to natural mpl ligand having a corresponding sequence. Preferably, the resulting molecules are those having additional glycosylation sites that are occupied by glycosyl chains upon expression in a mammalian cell (such as COS, CHO, and human cells).

In a first embodiment, the subject invention relates to analogs of mpl ligand comprising an amino acid sequence which includes at least one added, at least one deleted, and/or at least one added and deleted, site for glycosylation as compared to corresponding natural sequence mpl ligand. The added or deleted site(s) for glycosylation may result in a greater or lesser number of carbohydrate chains, and higher or lower sialic acid content, than corresponding natural sequence mpl ligand, particularly human mpl ligand. A combination of a deletion of one site and addition of another site would result in no net change in the number of sites, but rather, a change in location and/or type of site. Such combined change analogs are also encompassed within this invention.

In another aspect of the above embodiment, the subject invention relates to mpl ligand analogs comprising amino acid sequences which include replacement of one or more N- or O-linked glycosylation sites with one or more non-naturally occurring sites. Thus, an N-linked site may be replaced with a different

N-linked site; an N-linked site may be replaced with an O-linked site; an O-linked site may be replaced with a different O-linked site; and/or an O-linked site may be replaced with an N-linked site. Replacement of one site with another site in essentially the same location may have the result of increasing the glycosylation efficiency at that site, or other effects. For example, evidence is provided herein that a Thr residue instead of a Ser residue may increase the glycosylation efficiency at O-linked sites.

The term "mpl ligand", as used herein, includes naturally occurring mpl ligand, truncations of naturally occurring mpl ligand as well as non-naturally occurring polypeptides having an amino acid sequence and glycosylation sufficiently duplicative of that of naturally occurring mpl ligand to allow possession of a biological activity of specifically stimulating growth, development and/or production of megakaryocytes and/or platelets. Mpl ligand analogs based on at least amino acids 7-151 up to amino acids 1-332 of FIG. 1 are preferred.

In a preferred embodiment, mpl ligand is the product of the expression of an exogenous DNA sequence that has been transfected into a eukaryotic host cell; that is, in a preferred embodiment the mpl ligand is "recombinant mpl ligand". The preferred eukaryotic host is mammalian, particularly preferably CHO cells. Recombinant mpl ligand is advantageously produced according to the procedures described herein and in the publications cited herein regarding cloning and expression of mpl ligand.

Some additional preferred mpl ligand molecules have the following amino acid sequences, based on FIG. 1 herein:

| | |
|---|---|
| mpl ligand 1-332 | amino acids 1-332 of FIG. 1 |
| mpl ligand 1-199 | amino acids 1-199 of FIG. 1 |
| mpl ligand 1-191 | amino acids 1-191 of FIG. 1 |
| mpl ligand 1-183 | amino acids 1-183 of FIG. 1 |
| mpl ligand 1-174 | amino acids 1-174 of FIG. 1 |
| mpl ligand 1-163 | amino acids 1-163 of FIG. 1 |
| mpl ligand 1-153 | amino acids 1-153 of FIG. 1 |
| mpl ligand 1-152 | amino acids 1-152 of FIG. 1 |
| mpl ligand 1-151 | amino acids 1-151 of FIG. 1 |
| mpl ligand 7-332 | amino acids 7-332 of FIG. 1 |
| mpl ligand 7-199 | amino acids 7-199 of FIG. 1 |
| mpl ligand 7-191 | amino acids 7-191 of FIG. 1 |
| mpl ligand 7-183 | amino acids 7-183 of FIG. 1 |
| mpl ligand 7-174 | amino acids 7-174 of FIG. 1 |
| mpl ligand 7-163 | amino acids 7-163 of FIG. 1 |
| mpl ligand 7-153 | amino acids 7-153 of FIG. 1 |
| mpl ligand 7-152 | amino acids 7-152 of FIG. 1 |
| mpl ligand 7-151 | amino acids 7-151 of FIG. 1 |

It should be noted, for example, that mpl ligand 1-183, 1-191, 7-183, and 7-191 encompass one or two additional naturally-occurring glycosylation sites on the C-terminus thereof, as compared to shorter sequences. In each of the above cases, Met-Lys may further be included in the N-terminus thereof.

The *in vitro* specific activities referred to herein are measurements of relative *in vitro* specific activities and are not measurements of absolute *in vitro* specific activities. For the purposes of this application, the specific activities are used only to compare relative activities of mpl ligand analogs that have been assayed using the same assay, using the same conditions including the same internal standard, and having the same analysis of the data used to calculate specific activity, etc.

As used herein the phrases "analog of mpl ligand" or "mpl ligand analog" refer to mpl ligand with one or more changes in the amino acid sequence of mpl ligand which result in a change in the type (N- or O-linked, which may affect the amount of carbohydrate attached), number, or location of sites for carbohydrate attachment. In a preferred embodiment, the change in glycosylation site(s) results in a change in the number of glycosyl chains attached to the mpl ligand molecule. In a particularly preferred embodiment, the change in glycosylation site(s) adds at least one (generally 1-6, preferably 1-5, particularly preferably 2-4) glycosyl chains, and most preferably the chain(s) is(are) added via N-linkage. In another particularly preferred embodiment, the mpl ligand analog retains at least equivalent biological activity *in vivo* as compared to natural sequence mpl ligand (e.g., human mpl ligand) and may possess substantially higher activity *in vivo*, as measured in assays for biological activity. Such assays include those that detect megakaryocyte or platelet production.

To prepare such analogs of mpl ligand, preferably they are generated by site-directed mutagenesis resulting in additions, deletions, or substitutions of amino acid residues that add, eliminate or alter sites that are available for glycosylation. By "altered" is meant that a site has been deleted while another has been added at the same or another location as the deleted site. However, as is appreciated by those skilled in the art, other methods could result in a gene encoding the same amino acid sequence, and such methods are encompassed herein. The resulting analogs may have fewer or more (preferably more) attached carbohydrate chains than natural human/recombinant mpl ligand.

Addition of one or more carbohydrate (i.e.,

glycosyl) chains to mpl ligand is one important object of this invention. Mpl ligand analogs having more carbohydrate chains than those found in the corresponding naturally-occurring amino acid sequence (e.g., 1-332 or 1-174, etc.) are generated by adding glycosylation sites which do not perturb the secondary or tertiary conformation in a way that would substantially reduce biological activity. As used herein the "naturally-occurring" mpl ligand refers to an amino acid sequence having the corresponding number of amino acids as the relevant analog, even if the particular length of mpl ligand species is not actually expressed in the native species. Advantageously, the analog of mpl ligand has up to 6 additional sites for N-glycosylation or O-glycosylation, resulting in the addition of from 1 up to 6 additional N-linked or O-linked carbohydrate chains (or a combination thereof).

For example, a Pro at position 30 is replaced by an Asn and a Val at position 32 is replaced by a Thr to give the sequence Asn-Glu-Thr, which serves as a new site for N-glycosylation (analog N4 below; *see* Table 1).

Analogs may also be constructed which have two or more additional N-linked chains by combining mutations; for example, analogs N4 and N10 described in Table 1 may be combined to yield an analog with two additional sites for carbohydrate addition (i.e., analog N15 in Table 1). In a like manner analogs with three or more added chains can be constructed. As will be appreciated by those skilled in the art, the subject invention includes many other analogs of mpl ligand having different sites for glycosylation (in terms of number, type or location of site). The mpl ligand analogs of this invention are in all cases particularly preferably based on mpl ligand having a human amino acid sequence (see FIGs. 1 and 2); however, analogs based on mpl ligand sequences from other species (e.g., dog, pig,

monkey, mouse or rat) are also contemplated herein.

Insertions of amino acids to create glycosylation sites are also contemplated. For example, a Glu at position 57 is replaced by a Thr and Asn is inserted immediately after Met at position 55 as follows:

-Gln-Met-Glu-Glu-Thr-
54 55 56 57 58
→
-Gln-Met-Asn-Glu-Thr-Thr-
54 55 55' 56 57 58

This adds a new glycosylation site (amino acids 55', 56, and 57). See analog N23 below.

Also included within the analogs of this invention are analogs which have one or more amino acids extending from the carboxy terminal end of mpl ligand wherein the carboxy terminal extension provides at least one additional carbohydrate site. The carboxy terminus of mpl ligand will vary depending upon the particular form of mpl ligand used (e.g., mpl ligand 1-332 amino acids, or mpl ligand 1-163 amino acids). An additional carbohydrate site may be added to the carboxy terminus of an mpl ligand species by adding amino acids to the carboxy terminus, such amino acids containing one or more N- or O-linked glycosylation sites.

Tables 1 and 6 list some exemplary mpl ligand analogs which have additional sites for N-linked carbohydrate chains. The analogs have the sequence Asn-X-Ser or Asn-X-Thr included at various positions in the human mpl ligand polypeptide chain based on the human amino acid sequences to create N-linked sites. Tables 4 and 7 list those analogs which add at least one additional N-linked carbohydrate chain, as evidenced by the migration of the glycoproteins on SDS gels (*see*, Example 6 and FIGs. 3, 5, 6, 7, 9, 10, 12, and 13). Note that these Tables also include some truncated

species that are not "analogs" as defined herein (i.e., N1, N16, N17, and N31). These are listed in the Tables to show how various truncated species were prepared.

Also encompassed by the present invention are DNA sequences encoding the mpl ligand analogs disclosed herein, preferably those encoding analogs having additional sites for N-linked chains. Procedures used to introduce changes into the mpl ligand DNA sequence for the purpose of creating, deleting and/or altering attachment sites for carbohydrates are disclosed in Examples 4 and 14.

These mpl ligand analogs can be the product of expression of an exogenous DNA sequence, i.e., produced through recombinant DNA technology, they can be chemically synthesized products or they may be produced by combined methods. An exogenous DNA sequence comprises cDNA, genomic DNA or chemically synthesized DNA encoding an mpl ligand analog. Recombinant DNA plasmids and eukaryotic host cells useful for the expression of such analogs are also provided. Expression vectors include any vector which is capable of expressing cloned DNA sequences in a eukaryotic host cell, particularly those vectors used for expression in COS and CHO cells. Examples of such vectors include plasmids pDSRα and pDSRα2, see *Mol. Cell. Biol.* 8:466-472 (1988); WO 91/13160 (1991); and WO 90/14363 (1990). The cultivation of COS and CHO host cells expressing mpl ligand analogs was carried out using standard procedures known to those skilled in the art.

Changing the number, type, location, or amount of carbohydrate chains attached to mpl ligand may confer advantageous properties such as increased solubility, greater resistance to proteolysis, reduced immunogenicity, increased serum half-life, and increased or altered biological activity.

Conditioned media from COS cells expressing

mpl ligand analogs N2 - N15 (N1 is human mpl ligand 1-174; *see* FIG. 2) were analyzed for *in vitro* biological activity and the results shown in Table 4.

Conditioned media from COS cells expressing mpl ligand analogs/truncations N15-N40 were analyzed for *in vitro* biological activity and the results are shown in Table 7.

*In vivo* biological activity results for various forms are presented in FIG. 11 (see Example 13).

Another embodiment of the invention relates to mammalian (e.g., Chinese Hamster Ovary, CHO) host cells which preferentially synthesize mpl ligand or analogs of mpl ligand having greater than a specific number of sialic acids per molecule, e.g. greater than that found in mpl ligand 1-332, 1-199, 1-191, 1-183, 1-174, 1-163, 1-153, 1-152, or 1-151 produced naturally or recombinantly in a eukaryotic cell. In vitro activities of analogs N4 and N15, along with full-length and various truncated species expressed in CHO cells are shown in Table 5.

The sialic acid content of the mpl ligand molecule may affect its *in vivo* biological activity. For example, tetraantennary (four-branched) N-linked oligosaccharides most commonly provide four possible sites for sialic acid attachment while bi- and triantennary oligosaccharides, which can substitute for the tetraantennary form at asparagine-linked sites, commonly have at most only two or three sialic acids attached. O-linked oligosaccharides commonly provide two sites for sialic acid attachment. Thus, mpl ligand molecules with N-linked carbohydrate substituted for O-linked carbohydrate can accommodate two additional sialic acids per chain provided the N-linked oligosaccharides are tetraantennary. Mammalian cell cultures are screened for those cells that preferentially add tetraantennary chains to recombinant

mpl ligand, thereby maximizing the number of sites for sialic acid attachment.

Dihydrofolate reductase (DHFR) deficient Chinese Hamster Ovary (CHO) cells are a commonly used host cell for the production of recombinant glycoproteins including recombinant mpl ligand.

Compositions comprising a therapeutically effective amount of an mpl ligand analog in accordance with this together with a suitable diluent, adjuvant and/or carrier useful in mpl ligand therapy are further encompassed by this invention. A "therapeutically effective amount" as used herein refers to that amount which provides therapeutic effect for a given condition and administration regimen.

The present compositions can be systemically administered parenterally. Alternatively, the compositions may be administered intravenously or subcutaneously. When systemically administered, the therapeutic compositions for use in this invention may be in the form of a pyrogen-free, parenterally acceptable aqueous solution. The preparation of such pharmaceutically acceptable protein solutions, with due regard to pH, isotonicity, stability and the like, is within the skill of the art. The specific route chosen will depend upon the condition being treated. The administration of mpl ligand or mpl ligand analogs is preferably done as part of a formulation containing a suitable carrier, such as human serum albumin, a suitable diluent, such as a buffered saline solution, and/or a suitable adjuvant. The required dosage will be in amounts sufficient to raise the platelet levels of patients and will vary depending upon the severity of the condition being treated, the method of administration used and the like.

The conditions to be treated by the methods and compositions of the present invention are generally

those which involve an existing megakaryocyte/platelet deficiency or an expected megakaryocyte/platelet deficiency in the future (e.g., because of planned surgery). Such conditions will usually be the result of a deficiency (temporary or permanent) of active mpl ligand *in vivo*. The generic term for platelet deficiency is thrombocytopenia, and hence the methods and compositions of the present invention are generally available for treating thrombocytopenia.

Thrombocytopenia (platelet deficiencies) may be present for various reasons, including chemotherapy, bone marrow transplants, and other therapy with a variety of drugs, radiation therapy, surgery, accidental blood loss, and other specific disease conditions. Exemplary specific disease conditions that involve thrombocytopenia and may be treated in accordance with this invention are: aplastic anemia, idiopathic thrombocytopenia, metastatic tumors which result in thrombocytopenia, systemic lupus erythematosus, splenomegaly, Fanconi's syndrome, vitamin B12 deficiency, folic acid deficiency, May-Hegglin anomaly, Wiskott-Aldrich syndrome, and paroxysmal nocturnal hemoglobinuria. Also, certain treatments for AIDS result in thrombocytopenia (e.g., AZT). Certain wound healing disorders might also benefit from an increase in platelet numbers.

With regard to anticipated platelet deficiencies, e.g., due to future surgery or future thrombocytopenia-inducing therapy, an mpl ligand analog of the present invention could be administered several days to several hours prior to the need for platelets. With regard to acute situations, e.g., accidental and massive blood loss, an mpl ligand analog could be administered along with blood or purified platelets.

The dosage regimen involved in a method for treating the above-described conditions will be

determined by the attending physician, considering various factors which modify the action of drugs, e.g. the age, condition, body weight, sex and diet of the patient, the severity of any infection, time of administration and other clinical factors. Generally, the daily regimen should be in the range of 0.01-1000 micrograms of mpl ligand analog per kilogram of body weight, preferably 0.1-10 micrograms per kilogram of body weight.

The therapeutic methods, compositions and polypeptides of the present invention may also be employed, alone or in combination with other cytokines, soluble mpl (i.e., mpl ligand) receptor, hematopoietic factors, interleukins, growth factors or antibodies in the treatment of disease states characterized by other symptoms as well as platelet deficiencies. It is anticipated that an mpl ligand analog molecule will prove useful in treating some forms of thrombocytopenia in combination with general stimulators of hematopoiesis, such as IL-3 or GM-CSF. Other megakaryocytic stimulatory factors, i.e., meg-CSF, stem cell factor (SCF), leukemia inhibitory factor (LIF), oncostatin M (OSM), or other molecules with megakaryocyte stimulating activity may also be employed with mpl ligand. Additional exemplary cytokines or hematopoietic factors for such co-administration include IL-1 alpha, IL-1 beta, IL-2, IL-3, IL-4, IL-5, IL-6, IL-11, colony stimulating factor-1 (CSF-1), GM-CSF, granulocyte colony stimulating factor (G-CSF), EPO, interferon-alpha (IFN-alpha), IFN-beta, or IFN-gamma. It may further be useful to administer, either simultaneously or sequentially, an effective amount of a soluble mammalian Mpl receptor, which appears to have an effect of causing megakaryocytes to fragment into platelets once the megakaryocytes have reached mature form. Thus, administration of mpl ligand analog (to

enhance the number of mature megakaryocytes) followed by administration of the soluble mpl receptor (to inactivate the analog and allow the mature megakaryocytes to produce platelets) is expected to be a particularly effective means of stimulating platelet production. The dosage recited above would be adjusted to compensate for such additional components in the therapeutic composition. Progress of the treated patient can be monitored by conventional methods.

Additional modifications of the analogs of this invention may also be carried out, e.g., to increase activity, stability, half-life, etc. For example, pegylation (poly- or mono-) could be added to the mpl ligand analog via amino groups on the protein or via the carbohydrate groups. Also, fatty acids or other polymers could be attached to the protein or carbohydrate groups.

The following examples are offered to more fully illustrate the invention, but are not to be construed as limiting the scope thereof. The mpl ligand standard used in the bioassays employed in the Examples is a recombinant mpl ligand standard that was expressed in *E. coli*, refolded into an active conformation and purified. Thus, only relative specific activities are being measured.

## EXAMPLE 1

### Construction of Mpl Ligand 1-174

Human mpl ligand gene encoding amino acids 1-174 (starting with S-P-A-P-P-A...) of FIG. 2 was generated from a human fetal liver cDNA library (Bartley et al, *Cell* **77**: 1117-1124 (1994) by polymerase chain reaction (PCR). The 5' PCR primer encoded the amino terminus of human mpl ligand, an XbaI site, and an

optimized Kozak sequence. The 3' primer contained a termination codon and a SalI restriction site. The amplified DNA fragment was digested with XbaI and SalI then ligated to XbaI and SalI cut pDSRα2. The resultant plasmid, pDSRα2 mpl ligand 1-174 was used for mammalian cell expression. The sequence of the resulting gene (including the signal peptide) is shown in FIG. 2.

Plasmid DNA containing mpl ligand 1-174 was digested with XbaI and SalI restriction enzymes, the resulting DNA fragments were subjected to agarose gel electrophoresis, and the 605 nt mpl ligand 1-174 DNA fragment was isolated from the gel using a GeneClean™ kit and procedures supplied by the manufacturer (BIO 101, Inc.). Plasmid pDSRα2 as described in WO 90/14363 (1990) was also digested with XbaI and SalI restriction enzymes and the vector fragment was recovered. Ligation of the two fragments results in pDSRα2 (mpl ligand 1-174).

## EXAMPLE 2

### Expression of Mpl Ligand 1-174 in CHO Cells and Purification

Dihydrofolate reductase deficient ($DHFR^-$) Chinese Hamster Ovary (CHO) cells were transfected with pDSRα2-mpl ligand 1-174. A 100 mm tissue culture dish was plated with $1x10^6$ CHO $DHFR^-$ cells grown in CHO $D^-$ medium (DMEM, 10% Fetal bovine serum, 1% penicillin/streptomycin/glutamine, 1% nonessential amino acids(Gibco) and 1% HT supplement (Gibco)) the day before transfection. Four transfections were performed. For each transfection, plasmid DNA (50 ug) was linearized by digesting with Pvu I and Buffer H (Boehringer Mannheim). A DNA precipitate was then formed and added to the plates dropwise as per the

Mammalian Cell Transfection Kit (Speciality Media). After 24 hours in a tissue culture incubator the medium was replaced with fresh CHO D- medium. Twenty four hours later the cells were split into 96 well tissue culture plates with 100 ul of CHO select medium (D-MEM, 5% dialyzed fetal Bovine serum, 1% penicillin/streptomycin/glutamine, 1% nonessential amino acids (Gibco)) per well and transformants were selected. Medium was changed weekly until colonies appeared. After two weeks, mpl ligand expression was screened for using the 32D cell proliferation assay described below (see Example 9). Those clones expressing in excess of $1x10^5$ units/ml were expanded and frozen in cryogenic storage. One clone was expanded for roller bottle production and approximately 8 liters of conditioned medium was produced.

Plasmid pDSRα2 containing mpl ligand 1-174 cDNA was transfected into DHFR-deficient CHO cells as explained above. Two liters of serum-free CHO cell conditioned medium (50% D-MEM, 50% HAMS-F12, 1% penicillin/ streptomycin/glutamine, 1% nonessential amino acids(Gibco)) from roller bottles seeded with CHO cells expressing mpl ligand 1-174 was concentrated 15 fold using a 2L Amicon Model 2000 stirred cell and a 10,000 dalton molecular weight cut-off membrane (YM10, Amicon). Forty-five milliliters of concentrated conditioned medium was then loaded directly onto a 4 ml hu-MPL-X affinity column at a flow rate of 0.4 ml/min using a Pharmacia FPLC. The affinity column was constructed by coupling 1.5-2.5 milligrams of Mpl-X (the soluble extra-cellular domain of the mpl receptor) per milliliter of Pharmacia CNBr activated Sepharose resin as recommended by the manufacturer. After loading, the column was washed with 16 ml of phosphate buffered saline (PBS; 10 mM $Na \cdot PO_4$ pH 6.8/150 mM NaCl) and then 24 ml of 10 mM Tris, pH 8.0 / 1M NaCl. Mpl ligand

(1-174) was eluted with 40 ml of 20 mM CAPS (3-[Cyclohexylamino]-1 propanesulfonic acid) pH 10.5 / 1M NaCl / 5 mM CHAPS(3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate) in 6 ml fractions. The second fraction yielded a single band on a 14% SDS gel. This material was concentrated and buffer exchanged against a saline solution of 0.9% NaCl and was biologically active *in vitro* and *in vivo*. Other forms of CHO cell expressed mpl ligand were purified in a similar manner.

## EXAMPLE 3

### *In vivo* Biological Activity of Recombinant Human Mpl Ligand

Platelet counts from mice treated with various forms of mpl ligand were measured. CHO-derived mpl ligand 1-332, 1-174, 1-163, and 1-153 were produced and purified by Mpl-receptor affinity chromatography. *E. coli*-derived Met-Lys-mpl ligand 1-332, Met-Lys-mpl ligand 1-174, Met-Lys-mpl ligand 1-163 and Met-Lys-mpl ligand 1-153 were produced and purified by conventional chromatography.

FIG. 4 shows platelet counts from mice treated with various forms of CHO cell-derived (solid lines) or *E. coli*-derived (dashed lines) recombinant human mpl ligand. Normal, female Balb/c mice were injected subcutaneously with the indicated concentration of mpl ligand for 5 consecutive days. Test bleeds from a small lateral cut in a tail vein were collected 24 hours after the last injection. Blood cell analyses were performed with a Sysmex electronic blood cell analyzer (Baxter Diagnostics, Inc. Irvine, CA). Data are represented as the mean of determinations of 4

animals, +/- standard error of the mean. Other blood cell parameters such as total white blood cell counts or red blood cell counts were not affected by these treatments (data not shown).

The results indicate that CHO cell expressed forms of mpl ligand have an increased *in vivo* activity relative to the same forms of mpl ligand produced in *E. coli*. As described in Example 6, the CHO cell expressed forms of mpl ligand all contain N and/or O-linked carbohydrate and the *E. coli* expressed mpl ligand forms do not. This indicates that the carbohydrate enhances the *in vivo* activity of mpl ligand. The increased *in vivo* activity conferred by the carbohydrate may be a result of increased circulatory half life, increased stability or a combination of both.

## EXAMPLE 4

### Construction of Mpl Ligand Analogs N2-N15

Procedures for generating additional glycosylation sites for mpl ligand are described below.

The following oligonucleotide primers were synthesized for use in *in vitro* mutagenesis to prepare analogs N2-N14 (see Table 1 for the structures of these analogs):

| | | |
|---|---|---|
| N2 - | CCCATGTCAATCACAGCAGACT | SEQ ID NO.: 5 |
| N3 - | CTTCACAGCAACCTGAGCCAGT | SEQ ID NO.: 6 |
| N4 - | CAGTGCAACGAGACCCACCCTTTG | SEQ ID NO.: 7 |
| N5 - | GCCTACAAATGTCACGCTGCCTGCT | SEQ ID NO.: 8 |
| N6 - | CCCACTTGTAACTCATCCCTC | SEQ ID NO.: 9 |
| N7 - | CAACTGAACGCCACTTGTCTCTCA | SEQ ID NO.: 10 |
| N8 - | ACTTGTCTCAACTCCACCCTGGGGGA | SEQ ID NO.: 11 |
| N9 - | CTCCTGGGGAACCTTTCTGGA | SEQ ID NO.: 12 |
| N10 - | GACCACAAATCACACCGATCCCAAT | SEQ ID NO.: 13 |

N11 - ACCCTTTGTCTACAAATGTCACGCTGCCTGCT SEQ ID NO.: 14
N12 - TCTCTCAAACCTCACGGGGGAGCTT SEQ ID NO.: 15
N13 - TGGAAAAATCAGACGGAGGAGAC SEQ ID NO.: 16
N14 - TGGAGGAGAACAAGACACAGGACAT SEQ ID NO.: 17

To construct m13mp18 mpl ligand 1-174, the gene of FIG. 2 was introduced into XbaI and SalI restriction enzyme digested m13mp18 DNA. Single stranded DNA was recovered from supernatants of *E. coli* strain RZ1032 infected by m13mp18(mpl ligand 1-174) as described by Kunkel et al., *Methods in Enzymol.* **154**:367 (1987) and Messing, *Methods in Enzymol.* **101**:20 (1983). For *in vitro* mutagenesis approximately 0.5 µg of single-stranded DNA and 0.125 pmole of one of the synthetic primers described above were mixed with 6 µl of buffer (250 mM Tris pH 7.8, 50 mM $MgCl_2$, 50 mM dithiothreitol and 1% Bovine serum albumin (BSA-Pharmacia)). The primers were previously kinased with ATP and T4 polynucleotide kinase prior to addition. For annealing of the primer to the template, the reaction volume was adjusted to 10 µl with water, the mixture was heated to 65°C for 5 minutes and then allowed to cool to room temperature. For the elongation reaction 2.5 µl of each of dTTP, dATP, dGTP and dCTP and 1 ml ATP (all at 10 µM) were added, followed by 1 µl (1 unit) of *E. coli* DNA polymerase (Klenow fragment) and 1 µl (1 unit) of T4 DNA ligase. The mixture was then incubated overnight at 14°C and used to transform *E. coli* JM 109 (Yanisch-Perron et al. *Gene* **33**, 103 (1985)) as described (Messing, *supra*).

To identify mutant clones by differential hybridization, plaques on nutrient agar were transferred to Gene Screen filters (New England Nuclear). The DNA was cross-linked to filters by irradiating them in a UV Stratalinker Model 1800 using the auto cross-link mode (Stratagene). They were then incubated for one hour in

6x SSC(0.9M NaCl/0.09M Na·citrate) containing 1% SDS at 60°C. For the hybridization, the oligonucleotide primer above (8 pmoles) was end-labeled with T4 polynucleotide kinase and $\gamma$ $^{32}$P-labeled ATP and incubated with the filters overnight in 6x SSC, 0.5% SDS and 125 ug/ml herring sperm DNA. The hybridization temperatures were chosen according to estimates of oligonucleotide melting points. Generally the hybridization temperature was approximately 10°C less than the melting point. The next day, the filters were washed two times with 6X SSC/1% SDS at hybridization temperature followed by two washes with 6x SSC at hybridization temperature and subjected to autoradiography. If necessary, the filters were then washed with 6x SSC at increasing temperatures until little or no hybridization was detected to plaques having the wild-type mpl ligand cDNA sequence. Clones that gave positive hybridization signals under these conditions were identified and retransfected into JM109 to isolate a pure clone. Dideoxy chain termination sequence analysis indicated that the mutations were present.

Double stranded m13 mpl ligand 1-174 DNAs carrying the desired changes were recovered from JM109 transfected cells with QIAGEN kits (Chatsworth CA.) using methods supplied by the manufacturer. The DNAs were digested with XbaI and SalI and the 605 bp mpl ligand DNA fragments were isolated. pDSR$\alpha$2 was digested with XbaI and SalI. The vector fragment was isolated and ligated to the mpl ligand fragments above. Recombinant plasmids were identified by restriction analysis. The resulting plasmids (designated mpl ligand 1-174-NX where NX is the analog number) contain DNA encoding mpl ligand analogs having altered amino acid residues at the indicated positions. The resultant plasmids were then sequenced again to confirm the presence of the desired mutations.

The analog N15 was constructed that had two additional N-linked glycosylation sites at positions 30 and 120. PDSRα2 mpl ligand 174-N4 containing Asn30 and Thr32 mutations was digested with XbaI and PstI restriction enzymes and the approximately 385 nt DNA fragment was isolated. PDSRα2 mpl ligand 174-N10 containing Asn120 and Thr122 mutations was digested with PstI and SalI restriction enzymes and the approximately 220 nt DNA fragment was isolated. pDSRα2 was digested with XbaI and SalI. The vector fragment was isolated and ligated to the mpl ligand fragments above. This resulted in PDSRα2 mpl ligand 174-N15 that contains Asn30, Thr32, Asn120 and Thr122 substitutions.

These general procedures were used to construct the mpl ligand analogs shown in Table 1. The DNA sequence changes for each of the analogs are shown; otherwise the oligonucleotide primers used for mutagenesis had sequences complementary to those of human mpl ligand.

TABLE 1

| Analog/ Species No. | Amino Acid Substitution | Sequence Changes |
|---|---|---|
| N1 | (1-174): $Pro^{175} \rightarrow Gly^{332}$ deleted | CCA→TGA (stop codon) |
| N2 | $Leu^{22} \rightarrow Asn^{22}$ | CCT→AAT |
| N3 | $Arg^{25} \rightarrow Asn^{25}$ | AGA→AAC |
| N4 | $Pro^{30}$, $Val^{32} \rightarrow Asn^{30}$, $Thr^{32}$ | CCA,GTT →AAC,ACC |
| N5 | $Pro^{38}$, $Leu^{40} \rightarrow Asn^{38}$, $Thr^{40}$ | CCT,CTG →AAT,ACG |
| N6 | $Leu^{86} \rightarrow Asn^{86}$ | CTC→AAC |
| N7 | $Gly^{82}$, $Pro^{83} \rightarrow Asn^{82}$, $Ala^{83}$ | GGA,CCC→AAC,GCC |
| N8 | $Ser^{87}$,$Leu^{89} \rightarrow Asn^{87}$,$Thr^{89}$ | TCA,CTC→AAC,ACC |
| N9 | $Gln^{92} \rightarrow Asn^{92}$ | CAG→AAC |
| N10 | $Ala^{120}$,$Lys^{122} \rightarrow Asn^{120}$,$Thr^{122}$ | GCT,AAG→AAT,ACC |
| N11 | $Pro^{36}$,$Pro^{38}$,$Leu^{40} \rightarrow$ $Ser^{36}$,$Asn^{38}$,$Thr^{40}$ | CCT,CCT,CTG→ TCT,AAT,ACG |
| N12 | $Ser^{88}Leu^{90} \rightarrow Asn^{88}$,$Thr^{90}$ | TCC,CTG→AAC,ACG |
| N13 | $Thr^{53}$,$Met^{55} \rightarrow Asn^{53}$,$Thr^{55}$ | ACC,ATG→AAT,ACG |
| N14 | $Thr^{58}$,$Ala^{60} \rightarrow Asn^{58}$,$Thr^{60}$ | ACC,GCA→AAC,ACA |
| N15 | $Pro^{30}$,$Val^{32}$,$Ala^{120}$,$Lys^{122} \rightarrow$ $Asn^{30}$,$Thr^{32}$,$Asn^{120}$,$Thr^{122}$ | CCA,GTT,GCT,AAG→ AAC,ACC,AAT,ACC |

Note: Analogs N2-N15 are synonymously referred to herein as analogs 2-15. Further, as used herein, for example, [Asn$^{22}$] mpl ligand means that an asparagine has been substituted for the amino acid at position 22 in the particular mpl ligand species being considered, which is preferably a human sequence having at least amino acids 7-151 of FIG. 1 (including the preferred human mpl ligand sequences set forth herein above). Thus, substitution of an asparagine residue for a leucine residue at position 22 of mpl ligand 1-174 (human sequence) yields an mpl ligand analog that may be represented by [Asn$^{22}$] mpl ligand 1-174.

Plasmids designated pDSRα2 1-174-NX (where NX is the analog number) were constructed by inserting mpl ligand DNA into pDSRα2. The expression vector pDSRα2 is generally described in WO 90/14363(1990). pDSRα2 mpl ligand 1-174-NX plasmids were made by digestion of pDSRα2 with XbaI and SalI. The vector fragment was isolated and ligated to the approximately 605 bp fragments containing the desired sequences.

## EXAMPLE 5

## Expression of Mpl Ligand and Mpl Ligands N1-N15 in COS Cells

cDNA clones of human mpl ligand and mpl ligand analogs described in Table 1 were transferred into COS-1 cells (ATCC No. CRL-1650) by electroporation. COS-1 cells were harvested from semi-confluent dishes, washed with medium (Dulbecco's modified essential medium containing 10% fetal bovine serum and 1% L-glutamine/penicillin/ streptomycin (Irvine Scientific)) and resuspended at 6 x 10$^6$ cells/ml. One half ml of cells was transferred to a 0.2 cm electroporation cuvette (Bio-Rad) and electroporated

with a BTX Electroporation System Electrocell Manipulator 600 at 650 uF and 130 volts on the low voltage setting with 50 µg of plasmid DNA encoding the mpl ligand analog. The electroporated cells were plated on 100 mm tissue culture dish in 10 ml of medium. Twelve to twenty four hours after plating the medium was replaced with 10 ml of fresh medium. The conditioned medium was collected 3 to 5 days after electroporation.

## EXAMPLE 6

### Characterization of Mpl Ligand and Mpl Ligands N1-N15

A. Determination of Carbohydrate Addition

A volume of supernatant containing approximately 30-60 ng mpl ligand or mpl ligand analog from COS cells transfected with mpl ligand analog cDNAs as described in Example 5 was immunoprecipitated overnight at room temperature with a rabbit anti-mpl ligand polyclonal antibody. In some cases where expression was low, a maximum volume of approximately 8-9 ml was used for immuno-precipitation. The antibody was raised to mpl ligand 1-163 that had been expressed and purified from *E. coli*. Thirty µl of 1:1 Protein A-Sepharose in phosphate buffered saline (PBS) containing 0.1% sodium azide was added to the immunoprecipitate and allowed to incubate for one hour at room temperature. The samples were centrifuged, washed with PBS and resuspended in SDS sample Buffer (0.125 M Tris-HCl pH 6.8/ 4% SDS/ 20% glycerol/ 10% β-mercaptoethanol/ 0.001% bromophenol blue). The samples were analyzed by 12% SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose and subjected to Western analysis as described (Burnette et al., *Anal. Biochem.* **112**:195-203 (1981); Elliott et al.,

*Gene* **79**:167-180 (1989)) using a mouse anti-mpl ligand monoclonal antibody raised to a synthetic mpl ligand peptide (e.g., corresponding to amino acid residues 47-62 of FIG. 1). The mpl ligand containing bands were visualized using an ECL kit (Amersham).

FIG. 5 shows that COS cell supernatants from cells transfected with analogs N4, N7 and N10 DNA revealed increased size compared to human sequence mpl ligand 174 (N1). FIG. 6 shows that COS cell supernatants from cells transfected with N13, N14 and N4 DNA also had increased size compared to human sequence mpl ligand. This increased size is indicative of an additional N-linked carbohydrate chain. N15 contains two additional N-linked glycosylation sites. FIG. 6 indicates that this analog has material with a size greater than analogs containing only 1 additional N-linked glycosylation. The sizes of the proteins were estimated from their mobility on SDS-PAGE relative to protein standards of known molecular weight. The estimated sizes of the larger bands calculated from FIG. 6 are shown in Table 2. This result indicates that N15 contains 2 additional N-linked chains. Western blot analyses of other selected analogs are also shown in FIG. 6.

TABLE 2

## N-Linked Carbohydrate Estimates

| Mpl Ligand Analog (1-174) | Molecular Weight (Da) | Molecular Weight Shift(Da) (Over Native) | No. of Potential N-Linked Chains (@4 KDa/Site) |
|---|---|---|---|
| N1 (Native) | 23500 | 0 | 0 |
| N4 | 28700 | 5200 | 1 |
| N7 | 27200 | 3700 | 1 |
| N10 | 27200 | 3700 | 1 |
| N13 | 26700 | 3200 | 1 |
| N14 | 28700 | 5200 | 1 |
| N15 | 33500 | 10000 | 2 |

An experiment was performed to show that the increased size of mpl ligand analogs is due to N-linked carbohydrate. COS cell conditioned medium containing mpl ligand was immunoprecipitated and washed with PBS as described above. To each tube was then added 10 µl 0.5% SDS and each sample was boiled for 3 minutes. Then the following components were added: 10.8 µl of 0.5M $NaPO_4$ pH 8.6, 5 ml of 7.5% nonidet P40 and 3 ul of 250 unit/ml N-glycanase (Genzyme). N-glycanase treatment removes N-linked carbohydrate. Samples were incubated for 6 hours at 37°C. The reaction was stopped by the addition of SDS-PAGE sample buffer and then subjected to SDS-PAGE Western analysis (12% acrylamide) using an anti-mpl ligand monoclonal antibody and an anti-mouse ECL Western Detection Kit (Amersham) as described above. An analysis of N-linked chains using this method is shown in FIG. 7 for human mpl ligand and mpl ligand analogs. Following treatment with N-glycanase the mobility on Western blot for N4, N7 and N10 was reduced to that of N1. As expected, treatment of N1 with N-glycanase had no effect on mobility because N1 has no N-linked

glycosylation sites. These results indicate that the increased size observed is due to addition of N-linked carbohydrate.

B. Analysis of O-linked carbohydrate on mpl ligand

To analyze the contribution of O-linked carbohydrate to human mpl ligand, various forms of the protein were purified from CHO cell conditioned media as described above. Each form received +/- treatment with O-glycanase (Glycopeptide alpha-N-acetylgalactos-aminidase, Oxford GlycoSystems). O-glycanase removes O-linked carbohydrate from glycoproteins. The *E. coli* expressed version of each form was used as an unglycosylated control. To resolve the difference in molecular weight contributed by O-linked carbohydrate, it was necessary to remove any N-linked carbohydrate first. Since the full length version, mpl ligand 1-332, contains N-linked carbohydrate, the CHO cell expressed full length samples received N-glycanase (peptide-N4-(N-acetyl-beta-glucosaminyl) asparagine amidase) treatment as described above for COS cell expressed mpl ligand analogs, except that the N-glycanase treatment was an overnight incubation.

Before proceeding with the O-glycanase treatment on full length (1-332) mpl ligand, the pH range of the sample was adjusted to pH 6.0-pH 7.0 with 1/15 volume of 100 mM acetic acid, pH 2.2. One microgram of protein was denatured by boiling for 3 minutes in SDS and incubated at 37°C for 60 minutes with 1 U/ml neuraminidase (sialidase, from *Arthrobacter urefaciens*, Boehringer Mannheim) in 1 mM calcium acetate, pH 6.8 and 20 mM sodium phosphate, pH 6.8.

Subsequent treatment with O-glycanase was done by adding 5 mU of enzyme in a final volume of 100 ul, followed by an overnight incubation at 37°C. Proteins

(0.2 ug/lane) were separated by SDS-PAGE (15% acrylamide). Following transfer to 0.2 um nitrocellulose and overnight incubation with anti-mpl ligand polyclonal antibody the mpl ligand proteins were visualized using an anti-rabbit ECL Western Detection Kit (Amersham).

FIG. 3 shows a Western blot of four different forms of human mpl ligand. Full length mpl ligand 1-332 is represented in lanes 1-3, mpl ligand 1-174 lanes 4-6, mpl ligand 1-163 lanes 7-9, and mpl ligand 1-153 lanes 10-12. Treatment with neuraminidase and O-glycanase, shown in lanes 2,5,8, and 11, reduced the molecular weight to that of unglycosylated materials, lanes 3,6,9, and 12. In every case the mobility increased to that of the unglycosylated version expressed in *E. coli*. These results indicate that the larger sized bands, in lanes 1,4,7, and 10 are due to O-linked carbohydrate. The molecular weight of each of the bands was estimated by comparing their mobilities to proteins of known molecular weight.

As seen in Table 3 which shows estimated molecular weights of the different proteins, the apparent shift in mobility could account for as many as 14 O-linked carbohydrate chains (assuming 950 daltons/chain) for mpl ligand 1-332, 9 chains for mpl ligand 1-174, 4 chains for mpl ligand 1-163, and 2 chains for mpl ligand 1-153. The sample run in lane 2 is full length mpl ligand 1-332. It would appear that this protein was degraded, possibly due to extended incubation in glycoenzymes at 37°C. Therefore, the *E. coli* expressed unglycosylated version in lane 3 was used to calculate the approximate molecular weight of O-linked carbohydrate added to CHO cell expressed mpl ligand 1-332.

These results are consistent with the presence of carbohydrate on all the CHO expressed forms of mpl

ligand tested. The presence of O-linked carbohydrate was confirmed for CHO cell expressed mpl ligand 1-332, 1-174, and 1-163 by direct analysis of monosaccharide composition. Sialic acids, GalNAc and Gal were released from glycoproteins by acid hydrolysis. The monosaccharides were detected by high pressure anion exchange chromatography and pulsed amperometric detection. All three sugars were detected in each of the forms of mpl ligand. This result is indicative of the presence of sialic acid containing O-linked carbohydrate. This data correlates with the *in vivo* data as seen in FIG. 4 where CHO cell expressed forms of mpl ligand were all more active *in vivo* than the equivalent forms expressed in *E. coli*. Thus, the presence of carbohydrate enhances the *in vivo* activity of mpl ligand.

TABLE 3

**O-Linked Carbohydrate Calculations**

| Mpl Ligand Form | O-Glycanase Treatment (+/-) | Molecular Weight (Da) | Molecular Weight Shift | # of Potential O-Linked Chains (@950 Da/Chain) |
|---|---|---|---|---|
| 1-332 | - | 54200 | 13600 | 14 |
| " | E. coli version | 40600 | | |
| 1-174 | - | 24600 | 8600 | 9 |
| " | + | 16000 | | |
| 1-163 | - | 18400 | 3900 | 4 |
| " | + | 14500 | | |
| 1-153 | - | 15200 | 2300 | 2 |
| " | + | 12900 | | |

EXAMPLE 7

Mpl Ligand ELISA Assay

Polyclonal antibody production-- New Zealand White rabbits were hyperimmunized over a period of three

months with recombinant human mpl ligand 1-163 produced in *E. coli*. Antisera from six rabbits exhibiting high antibody titers were pooled and specific anti-mpl ligand antibodies were affinity purified.

Affinity purification-- Recombinant human mpl ligand 1-163 was covalently attached to Actigel-ALD (Sterogene Bioseparations, Inc.) according to the manufacturer's instructions. An aliquot of the rabbit antisera pool was added to the mpl ligand affinity gel, and the slurry was agitated gently on a rocker platform overnight a 4-8°C. Unbound serum proteins were washed from the gel bed with PBS and specifically bound anti-mpl ligand antibodies were then eluted with ImmunoPure Gentle Ag/Ab Elution Buffer (Pierce Chemical Co.) Recovered antibodies were dialyzed against several changes of PBS, then the antibody solution was concentrated in an Amicon stirred cell ultrafiltration unit and the resultant antibody concentrate was the source of specific anti-mpl ligand antibodies subsequently used for well coating and enzyme conjugate preparations.

ELISA reagents--Immulon 4 Removawell Strips (Dynatech Laboratories, Inc.) were coated with affinity purified rabbit anti-mpl ligand antibodies. Affinity purified antibodies were diluted in 0.1 M sodium bicarbonate (freshly prepared pH about 8.2) to a concentration of 2.5 ug/ml. Each well received 100 ul of antibody and the plates were incubated for 24 hrs at room temperature in a sealed and humidified chamber. Then, 200 ul of a blocking solution consisting of 1% fetal bovine serum 5% sucrose in TEN (50 mM Tris 7.4/10 mM EDTA/150 mM NaCl) was added to each well and plates were incubated and additional 24 hrs at room temperature in a sealed and humidified chamber. Combined coating and blocking solutions were removed from the wells. An additional overcoating/blocking step was included: 300

ul of SuperBlock Blocking Buffer in PBS (Pierce Chemical Co.) was added to each well. After standing at room temperature for about 5 min. this solution was removed and the wells were allowed to air dry at room temperature for 24 hrs. The coated wells were stored in sealed plastic bags at 4-8°C until used in the mpl ligand ELISA.

Affinity purified anti-mpl ligand antibodies from a rabbit antisera pool were covalently coupled to horseradish peroxidase (HRPO) for use as the signal generating antibody. The affinity purified antibodies were derivatized with iminothiolane HCl (Fluka Chemical Corp.). Separately, HRPO was derivatized with N-succinimidyl 6-maleimidocaproate (Fluka Chemical Corp.). The two activated proteins were combined to permit covalent coupling. The reaction mixture was then chromatographed down a FPLC Superose 6 (Pharmacia) column to isolate the antibody:HRPO conjugate of the desired molecular weight (i.e. about 200 kD). Fractions containing the desired conjugate were combined and concentrated in a Centricon 30 (Amicon Division, W.R. Grace & Co.) and stored as a 50% glycerol solution at -20°C. This anti-mpl ligand Ab:HRPO concentrate was diluted into 2% fetal bovine serum in PBS for use in the ELISA. The final concentration of conjugate used in the ELISA was 250-500 ng/ml.

Recombinant human mpl ligand 1-163 produced in *E. coli* cells, was used for the preparation of standards. This mpl ligand was diluted into 2% fetal bovine serum (Sigma Chemical Co.) in TEN buffer containing 0.05% thimerosal as a preservative. Standards prepared contained 1.0, 0.5, 0.25, 0.125 and 0.062 ng/ml mpl ligand.

Assay-100 ul of mpl ligand standards or samples was added to wells then incubated for 18-24 hrs at room temperature in a sealed and humidified chamber.

The well contents and residual solution were then removed and the wells washed once with wash solution (0.05% Tween 20 in TEN buffer). Anti-mpl ligand Ab:HRPO conjugate solution (100 ul) was added to each well and then incubated for 2 hrs at room temperature in a sealed and humidified chamber. The contents of wells were removed then washed 4 times with 0.05% Tween 20 in TEN buffer.

For color development, 100 ul of TMB/peroxide substrate solution (Kirkegaard & Perry Solutions A & B mixed 1:1) was added and incubated for 20 min at room temperature. The reaction was stopped by addition of 100 ul stop solution (0.5 N sulfuric acid) and the absorbance was read at 450 nm on microtiter plate reader. Concentrations of mpl ligand in samples were calculated from a standard curve generated by using a curve fit program.

## EXAMPLE 8

### Biological Activity of Mpl Ligand 1-174 Analogs in a Short-Term Liquid Culture Megakaryocyte Assay

Analogs of mpl ligand 1-174 were prepared as described above and assayed for their ability to stimulate the growth of megakaryocytes in liquid culture. CD34 selected cells isolated from human leukapheresis units (Nichol et al., *Stem Cells* **12**:494-505 (1994)) were plated at $2x10^5$/ml in culture medium (IMDM / 1% Pen-Strep Glutamine / 1% Non-essential Amino Acids / 1% MEM Na-Pyruvate / 1% MEM Vitamins / 10% deionized BSA / 10% normal human AB plasma / 10 uM alpha-thiacylglycerol / 20 ug/ml L-Asparagine). In addition, 1.5 ul of COS-1 conditioned medium containing mpl ligand (1-174) or mpl ligand 1-174 analog was added to each well. The final volume was 15 ul in Terasaki-

style microtiter tissue culture plates (Vangard International). Cells were incubated at 37°C for eight days in humidified boxes in 5% $CO_2$, fixed directly to the culture wells with 1% glutaraldehyde, and then incubated with a monoclonal antibody cocktail consisting of anti-GPIb, anti-GPIIb, (Biodesign) and anti-GPIb (Dako, Carpinteria, CA). The immune reaction was developed with a streptavidin-β-galactosidase detection system (HistoMark, Kirkegaard and Perry). Megakaryocytes, identified by the darker color (blue in actual photographs), appear in FIG. 8.

Panels A and D of FIG. 8 are the positive and negative controls respectively. The well pictured in panel A received 37.5 pg of wild type mpl ligand 1-174 COS-1 conditioned medium and shows substantial megakaryocyte growth. Panel D received 1.5 ul of COS-1 mock conditioned medium and shows no growth. Panels B and C of FIG. 8 are mpl ligand 1-174 analogs N7 and N10 respectively. Panel B received 9.0 pg of mpl ligand COS-1 conditioned medium while panel C received 27 pg and both show excellent megakaryocyte growth.

This experiment indicates that the analogs of mpl ligand tested are capable of stimulating the growth of human megakaryocytes *in vitro*.

## EXAMPLE 9

### Biological Activity of Mpl Ligand 1-174 Analogs in an *In Vitro* Cell Proliferation Assay

Analogs of mpl ligand 1-174 were prepared as described above and assayed for their ability to stimulate the proliferation of 32D-mpl cells. To construct 32D-mpl cells, the full length human mpl receptor sequence (Vigon, I., et al., *PNAS* **89**:5640-5644 (1992)) was subcloned into an expression vector

containing the transcriptional promoter of Moloney Murine Sarcoma virus. Six ug of this construct and 6 ug of an amphotrophic retroviral packaging construct (Landau, N.R., Littman, D.R., *Journal of Virology* **66**:5110-5113 (1992)) were transfected into 3 x $10^6$ 293 cells using a $CaPO_4$ mammalian transfection kit (Stratagene). The same cells were retransfected after 2 days and again after 4 days. The day after the last transfection the 293 cells were cocultivated with the IL-3 dependent murine cell line (32D, clone 23; Greenberger et al., *PNAS* **80**:2931-2936 (1983)). After 24 hours, the 32D cells were rescued and banded in a BSA gradient (Path-o-cyte; Miles Inc.). Cells were expanded in 1 ng/ml murine IL-3 and then were selected for growth in 20% APK9 serum (Bartley et al., *Cell* **77**:1117-1124 (1994). Cells were sorted for cell surface expression of receptor by FACS using a polyclonal rabbit antipeptide (MPL) serum. These cytokine dependent murine 32D-mpl cells are responsive to mpl ligand. 32D-MPL cells were grown in MEM medium containing 10% Fetal Clone II Serum (Hyclone Laboratories) and 1.0 ng/ml muIL3 to a cell density of 1X$10^6$ cells/ml. Cells were collected by centrifugation (approx. 500XG) and washed twice in growth medium lacking muIL3 and resuspended at 1X$10^5$ cells/ml.

An extended twelve point mpl ligand standard curve was prepared using mpl ligand 1-163 and ranges from 5000 to 1 pg/ml. A volume of 100 ul of each dilution of standard mpl ligand or assay sample was added to appropriate wells of a 96 well microtiter tissue culture plate containing 100 ul of resuspended cells (10,000 cells/well) and incubated in a humidified incubator at 37°C and 10% $CO_2$. After 48 hours, 40 ul of MTS reagent (Aqueous Non-Radioactive Cell Proliferation Kit, Promega) was added to each well and 14-18 hours later the plates were read on a plate reader at 490 nM.

The *in vitro* activity in samples was calculated from a dose response curve for each sample. One unit was defined as the amount of mpl ligand in each sample required to give 50% of maximal stimulation. Specific activity was calculated by dividing the biological activity in units/ml by the mpl ligand concentration in ng/ml as determined by mpl ligand ELISA.

The specific biological activity of mpl ligand analogs transfected and expressed in COS cells is shown in Table 4. The effect of the amino acid substitutions on carbohydrate addition is also summarized. Purified human sequence mpl ligand has an *in vitro* activity that was 200-300 unit/ng as determined by the above-mentioned assays. It is apparent from Table 4 that mpl ligand analogs containing additional N-linked carbohydrate are expressed as well as native sequence mpl ligand even when they contain additional carbohydrate chains (as determined in Example 6, Section A) e.g., N4 and N10. Both of these analogs retained full *in vitro* biological activity also. Therefore the mpl ligand analogs containing N-linked carbohydrate can be expressed normally in mammalian cells and they can have normal or enhanced *in vitro* biological activity.

TABLE 4

| Mpl Ligand Form (Amino Acid Length) | Sequence | Number of N-linked chains (a) | Elisa (ng/ml) (b) | In Vitro Activity (units/ml) (c) | Specific Activity (units/ng) (d) |
|---|---|---|---|---|---|
| MOCK | NONE | 0 | <0.08 | <10 | <125 |
| N1 (174) | Native | NA | 25 | 5375 | 215 |
| N1 (174) | Native | 0 | 31.4 | 8800 | 280 |
| N1 (174) | Native | 0 | 31.75 | NA | NA |
| N2 (174) | N22 | 0 | NA | NA | NA |
| N3 (174) | N25 | NA | 1.85 | 636 | 344 |
| N4 (174) | N30T32 | 1 | 38 | 8830 | 232 |
| N4 (174) | N30T32 | 1 | 24 | NA | NA |
| N5 (174) | N38T40 | 0 | 1.2 | <10 | <8 |
| N6 (174) | N86 | 0 | 0.44 | <10 | <22 |
| N7 (174) | N82A83 | 0 to 1 | 6 | 2660 | 443 |
| N7 (174) | N82A83 | 0 to 1 | 4.7 | 3080 | 655 |
| N9 (174) | N92 | 0 | 10.5 | 1970 | 188 |
| N10 (174) | N120T122 | 1 | 20.4 | 5943 | 291 |
| N10 (174) | N120T122 | 1 | 33.7 | 9690 | 288 |
| N11 (174) | S36N38T40 | NA | <0.625 | <10 | <16 |
| N11 (174) | S36N38T40 | 0 | 1.3 | <10 | <8 |
| N13 (174) | N53T55 | 0 to 1 | 67 | 18000 | 269 |
| N14 (174) | N58T60 | 0 to 1 | 17.9 | 4850 | 271 |
| N15 (174) | N30T32N120T122 | 0 to 2 | 26 | 6420 | 247 |

NOTES

(a) The number of additional N-linked chains was estimated based upon the mobility of the analog polypeptides in SDS gels as described in Example 6.

(b) Quantities of mpl ligand analogs in CHO cell supernatants were determined by ELISA assay as described in the Examples.

(c) *In vitro* activity was determined by measuring stimulation of thymidine uptake in 32D cells dependent on mpl ligand for growth.

(d) Ratio of *in vitro* activity of mpl ligand analog as measured by proliferation assays to amount of mpl ligand analog measured by mpl ligand ELISA.

N.A. Not available.

EXAMPLE 10

Expression in CHO cells and Purification of Mpl Ligand 1-174, N4 and N15

pDSRα2 containing mpl ligand 1-174, N4 and N15 cDNA was transfected into DHFR-deficient CHO cells using the protocol described in Example 2 with the following modifications.

One transfection was performed for each analog. Three weeks after the transfection, mpl ligand expression was screened by mpl ligand ELISA. Three expressing clones for each form were frozen in cryogenic storage. The highest expressing clone for each analog was expanded for roller bottle production. For N4, 7.4 liters of conditioned medium (50% D-MEM, 50% HAMS-F12, 1% penicillin/ streptomycin/glutamine, 1% nonessential amino acids (Gibco)) was produced and for N15, 4.6 liters of conditioned medium was produced.

Serum-free CHO cell conditioned medium from roller bottles seeded with CHO cells expressing mpl ligand 1-174 (2.9 L), N4 (7.4 L), N15 (4.4 L) was concentrated 12-, 19-, and 12- fold respectively using a S1Y10 (10,000 dalton molecular weight cut-off) Amicon Spiral ultrafiltration cartridge. One hundred fifty milliliters of concentrated conditioned medium was then loaded directly onto a 3.3 ml hu-MPL-X (receptor) affinity column at a flow rate of 0.3 ml/min. The affinity column was constructed by coupling 1.0-1.5

milligrams of Mpl-X (the soluble extra-cellular domain of the Mpl receptor) per milliliter of Pharmacia CNBr activated Sepharose resin as recommended by the manufacturer. After loading, the column was washed with 30 ml of phosphate buffered saline (PBS; 10 mM $NaPO_4$ pH 6.8/150 mM NaCl) and then 60 ml of 10 mM Tris, pH 8.0 / 1M NaCl / 1 mM CHAPS. Mpl ligand 1-174 was eluted with 30 ml of 20 mM CAPS (3-[Cyclohexylamino]-1 propanesulfonic acid) pH 10.5 / 1M NaCl / 1 mM CHAPS(3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate).

Fractions were neutralized by adding 0.6 mL 1M Tris pH 7.0 to each eluted fraction. SDS-PAGE analysis showed an apparent "bleeding" of 1-174 mpl ligand during the 10 mM Tris, pH 8.0 / 1M NaCl 1 mM CHAPS wash. Elution fractions were analyzed by SDS-PAGE. Those fractions containing mpl ligand 1-174 were pooled. This affinity purification was then modified and repeated with the following changes: 0.5 mL/min load and elution, and the removal of the 10 mM Tris, pH 8.0 / 1M NaCl / 1 mM CHAPS wash.

All fractions containing the single mpl ligand band were concentrated using a YM10 (10,000 dalton molecular weight cut-off) membrane in a 50 mL stirred cell, switching to a centricon device. This 0.5 mL concentrate was loaded directly onto a PBS equilibrated Pharmacia Superdex 200 HR 10/30 gel filtration column at 0.25 mL/min collecting 0.25 mL fractions. All eluted fractions containing a single mpl ligand band (based on SDS-PAGE analysis) were pooled.

Other forms (N4 and N15) of CHO cell expressed mpl ligand were purified in a similar manner (two affinity purifications pooled and run on one Superdex 200 gel filtration column).

EXAMPLE 11

Determination of Carbohydrate Addition for CHO Cell Expressed N4 and N15

In order to determine whether N-linked carbohydrate was contained in the mpl ligand forms expressed in CHO cells, conditioned medium was analyzed by SDS-PAGE Western blot as described in Example 6 with the following modifications.

CHO D- Conditioned medium from roller bottles was used. Samples were loaded into the Centricon-10 centrifugal concentrators (Amicon, Beverly, MA.) and were spun at 6000 RPM for one hour in a Beckman J2-HS centrifuge using a fixed angle rotor (JA 20.1). A volume of concentrated sample containing approximately 100 ng of the mpl ligand analog was loaded on a SDS PAGE gel together with SDS sample buffer (described in Example 6.) *E. coli* expressed mpl ligand MK 1-174 containing no carbohydrate was also loaded. FIG. 9 shows differences in mobility that correlate with the expected amount of carbohydrate. The fastest mobility species, Met-Lys (1-174) *E. coli* mpl ligand, was followed by mpl ligand 1-174 (CHO), N4 (CHO), and N15 (CHO)in succession. See FIG. 9. The most likely explanation for size increases relative to unglycosylated mpl ligand is additional O-linked carbohydrate on mpl ligand 1-174 (CHO), additional O-linked carbohydrate and one additional N-linked oligosaccharide on N4 (CHO), and additional O-linked carbohydrate and two additional N-linked oligosaccharides on N15 (CHO).

In order to establish that the increase in molecular weight was indeed due to the addition of N-linked carbohydrate chains, the samples were treated with N-glycanase to remove any N-linked carbohydrate as described in Example 6. Each sample contained

approximately 100 ng of mpl ligand analog purified from conditioned medium.

Following treatment with N-glycanase the mobility of N4 (CHO) and of N15 (CHO) was reduced to that of mpl ligand 1-174 (CHO). Treatment of mpl ligand MK 1-174 (*E. coli*) or mpl ligand 1-174 (CHO) with N-glycanase did not affect mobility since neither form was expected to contain any N-linked carbohydrate. Comparison of N-glycanase treatment versus no treatment shows that the size difference for N4 corresponds to the size of one N-linked carbohydrate chain and the size difference for N15 corresponds to the size of two carbohydrate chains. Thus addition of N-linked glycosylation sites for these two mpl ligand forms resulted in additional N-linked carbohydrate when these species were expressed in CHO cells. See FIG. 10.

## EXAMPLE 12

### *In vitro* Biological Activity of Mpl Ligand Analogs made in CHO cells

Purified mpl ligand and analogs expressed and purified from in CHO cells or *E. coli* cells were analyzed for *in vitro* biological activity using the factor dependent cell line 32D-MPL and assay described in Example 9 except activity was calculated from a curve using mpl ligand 1-332 produced in CHO cells as standard. The specific *in vitro* biological activities of the various forms are shown in Table 5. It is apparent from this Table that the mpl ligand analogs containing additional carbohydrate, which are expressed in CHO cells have *in vitro* biological activity.

TABLE 5

## IN VITRO ACTIVITY OF MPL LIGANDS

| MPL LIGAND FORM | No. N-Linked Chains | IN VITRO ACTIVITY U/mg X10E6 |
|---|---|---|
| MK174( E. coli) | 0 | 13 |
| 1-163 (CHO) | 0 | 86 |
| 1-174 (CHO) | 0 | 85 |
| N4 (CHO) | 1 | 60 |
| N15 (CHO) | 2 | 92 |
| 1-332(CHO) | 6 | 41 |

EXAMPLE 13

*In vivo* Biological Activity of Mpl Ligand Analogs

Platelet counts from mice treated with various forms of mpl ligand were measured and the results are presented in FIG. 11. CHO-derived mpl ligand 1-332, 1-174, N4, and N15 were produced and purified by mpl-receptor affinity chromatography. *E. coli*-derived Met-Lys-mpl ligand 1-174, was produced and purified by conventional chromatography. The indicated

concentration of each form was administered subcutaneously into normal, female Balb/c mice once daily for 5 days. Test bleeds from a small lateral cut in a tail vein were collected 24 hours after the last injection. Blood cell analyses were performed with a Sysmex electronic blood cell analyzer (Baxter Diagnostics, Inc. Irvine, CA). Data are represented as the mean of determinations of 4 animals, +/- standard error of the mean. Other blood cell parameters such as total white blood cell counts or red blood cell counts were not affected by these treatments (data not shown).

All the forms stimulated increases in platelet counts. However the activities of the different forms varied. The relative *in vivo* activity was mpl ligand MK 1-174 (*E. coli*) < mpl ligand 1-174 (CHO) < N4 (CHO) < mpl ligand 1-332 (CHO) < N15 (CHO). The results indicate that addition of non-naturally occurring N-linked carbohydrate results in increased *in vivo* activity. It indicates further that increases in the amount of carbohydrate result in proportional increases in *in vivo* activity.

## EXAMPLE 14

### Construction of Mpl Ligand Analogs and Truncations N16-N40 by Overlap PCR

Analogs N16 to N40 (see Table 6 for the structures of these analogs) were constructed by overlap PCR (polymerase chain reaction) using a protocol adapted from Cheng et al., *PNAS* **91**, 5695 (1994). Typically one to two mutations were introduced in each construction.

The following oligonucleotide primers were synthesized for use to prepare analogs N16-N40:

| | |
|---|---|
| 5' F CCCTCTAGACCACCATGGAACTGACTGAATTGCTCCTC | SEQ ID NO.: 18 |
| 3' R (1-174) CCCGTCGACTCAGAGCTCGTTCAGTGTG | SEQ ID NO.: 19 |
| N16 - 3' R CCCGTCGACTCACTCCAACAATCCAGAAG | SEQ ID NO.: 20 |
| N17 - 3' R CCCGTCGACTTATCTGGCTGAGGCAGTGA | SEQ ID NO.: 21 |
| N18 - F CACGTCCTTAACAGCAGCCTGAGCCAGTG | SEQ ID NO.: 22 |
| N18 - R CACTGGCTCAGGCTGCTGTTAAGGACGTG | SEQ ID NO.: 23 |
| N19 - F CCCTTTGCCTAACGGTTCCCTGCTGCCTGCTGT | SEQ ID NO.: 24 |
| N19 - R ACAGCAGGCAGCAGGGAACCGTTAGGCAAAGGG | SEQ ID NO.: 25 |
| N20 - F TGCCTACACCTAACCTGTCGCCTGCTGTGGA | SEQ ID NO.: 26 |
| N20 - R TCCACAGCAGGCGACAGGTTAGGTGTAGGCA | SEQ ID NO.: 27 |
| N21 - F GGAAAACCAATATGTCGGAGACCAAGGCACA | SEQ ID NO.: 28 |
| N21 - R TGTGCCTTGGTCTCCGACATATTGGTTTTCC | SEQ ID NO.: 29 |
| N22 - F TGGGAGAATGGAACACCACGATGGAGGAGACC | SEQ ID NO.: 30 |
| N22 - R GGTCTCCTCCATCGTGGTGTTCCATTCTCCCA | SEQ ID NO.: 31 |
| N23 - F AAAACCCAGATGAACGAGACGACCAAGGCACA | SEQ ID NO.: 32 |
| N23 - R TGTGCCTTGGTCGTCTCGTTCATCTGGGTTTT | SEQ ID NO.: 33 |
| N24 - F CCCAGATGGAGAACACCTCGGCACAGGACAT | SEQ ID NO.: 34 |
| N24 - R ATGTCCTGTGCCGAGGTGTTCTCCATCTGGG | SEQ ID NO.: 35 |
| N25 - F CACGGGGACAAAACGGAACCACTTGCCTCTCA | SEQ ID NO.: 36 |
| N25 - R TGAGAGGCAAGTGGTTCCGTTTTGTCCCCGTG | SEQ ID NO.: 37 |
| N26 - F CAGGGCAGGAACACATCTCACAAGGATCCCA | SEQ ID NO.: 38 |
| N26 - R TGGGATCCTTGTGAGATGTGTTCCTGCCCTG | SEQ ID NO.: 39 |
| N27 - F GGGCAGGACCAACGCTAGCAAGGATCCCAAT | SEQ ID NO.: 40 |
| N27 - R ATTGGGATCCTTGCTAGCGTTGGTCCTGCCC | SEQ ID NO.: 41 |
| N29 - F pair1 CAGTGCAACGAGTCCCACCCTTGG | SEQ ID NO.: 42 |
| N29 - R pair1 CAAAGGGTGGGACTCGTTGCACTG | SEQ ID NO.: 43 |
| N29 - F pair2 GACCACAAATCACTCCGATCCCAA | SEQ ID NO.: 44 |
| N29 - R pair2 TTGGGATCGGAGTGATTTGTGGTC | SEQ ID NO.: 45 |
| N30 - F GTCCCCACCAACACCTCTCTAGTCCTC | SEQ ID NO.: 46 |
| N30 - R GAGGACTAGAGAGGTGTTGGTGGGGAC | SEQ ID NO.: 47 |
| N31 - 3' R CCCGTCGACTCACTTCAGAAGCCCAGAGCCAGT | SEQ ID NO.: 48 |
| N36(1) - F GAAAACCCAGAACGAGACCACCAAGGCACAG | SEQ ID NO.: 49 |
| N36(1) - R CTGTGCCTTGGTGGTCTCGTTCTGGGTTTTC | SEQ ID NO.: 50 |

| | | |
|---|---|---|
| N36(2) - F | CACCAAGGCACAGGACATTCTGGGAG | SEQ ID NO.: 51 |
| N36(2) - R | CTCCCAGAATGTCCTGTGCCTTGGTG | SEQ ID NO.: 52 |
| N37 - F | GAAAACCCAGATGAACGAGACCAAGGCACAG | SEQ ID NO.: 53 |
| N37 - R | CTGTGCCTTGGTCTCGTTCATCTGGGTTTTC | SEQ ID NO.: 54 |
| N38 - F | GTCCCCACCAACACCACTCTAGTCCTC | SEQ ID NO.: 55 |
| N38 - R | GAGGACTAGAGTGGTGTTGGTGGGGAC | SEQ ID NO.: 56 |

F = Forward

R = Reverse

Constructions that introduce one new glycosylation site were performed in two successive steps. In step 1, two reactions were performed using 4 different oligonucleotides. These oligos included a 5' forward primer, a reverse mutagenic primer, a forward mutagenic primer(usually complementary to the reverse mutagenic primer) and a reverse 3' primer. The reverse 3' primer contained sequences that introduced stop codons followed by SalI restriction sites. Stop codons were introduced at positions 175, 184, 192, and 200. Thus, forms of lengths 1-174, 1-183 (N16), 1-191 (N17), and 1-199 (N31) could be made. PCR1 used template DNA (pDSRα2 containing mpl ligand 1-174 sequences or full length mpl ligand 1-332 sequences), the 5' forward primer and the reverse mutagenic primer. PCR2 used template DNA, the 3' reverse primer and the forward mutagenic primer. The two PCR reactions were then performed and the amplified DNA fragments were separated by agarose gel electrophoresis. Small pieces of agarose containing DNA fragments of the correct size were excised from the gel.

The DNA fragments from PCR1 and PCR2 were combined together and a third PCR reaction was performed using only the 5' forward and 3' reverse primers. Thus, a full length DNA segment containing the desired mutations inserted into mpl ligand was amplified.

The amplified fragments were again separated by agarose gel electrophoresis, the correct sized DNA fragment was purified using a Geneclean™ kit and procedures supplied by the manufacturer (Bio 101, Inc.). The purified DNA was digested with XbaI and SalI, then it was purified again using the Geneclean™ kit. The fragment was then ligated into XbaI and SalI cut pDSRα2. Ligated DNA was precipitated with 2 volumes of ethanol in 0.3M NaOAc pH 5.2 in the presence of carrier tRNA and transformed into *E. coli*. Clones were tested by restriction analysis and agarose gel electrophoresis to identify those containing the correctly sized DNA inserts. Purified plasmid DNA was then prepared and the mpl ligand insert was sequenced to confirm the presence of the desired mutations and to ensure that no additional amino acid changes were introduced.

In several cases, two or more mutations were combined simultaneously, i.e., see N29, N33, N34, N35, N39 and N40. This could be done by introducing a new substitution into DNA already containing a change. For example, N33 was made by introducing the N23 changes into N15. In this case the procedure above was performed by using N23 mutagenic primers and the N15 template DNA.

In another strategy, two changes could be introduced simultaneously into template DNA. The template DNA could contain natural sequences or could contain sequences encoding mpl ligand forms already containing changes. In these cases step 1 involved 3 PCR reactions and 6 oligos. The oligos included a 5' forward primer, 2 pairs of forward and reverse mutagenic primers, and a reverse 3' primer. Each pair of primers was complementary to each other and contained sequences designed to introduce one new glycosylation site.

PCR1 included template DNA, the 5' forward primer and the reverse mutagenic primer from pair 1. PCR2 included template DNA, the forward mutagenic primer

from pair 1 and the reverse mutagenic primer from pair 2 where pair 2 primers are 3' to pair 1 primers. PCR3 included template DNA, the forward mutagenic primer from pair 2 and the reverse 3' primer.

DNA fragments from each PCR reaction were separated by agarose gel electrophoresis and excised as before. The 3 DNA fragments were then combined together and amplified by PCR again using only the 5' forward and 3' reverse primers.

The DNA segment encoding the entire gene of interest with sequences containing two new glycosylation sites was then purified, cut with XbaI and SalI, and ligated into XbaI and SalI cut pDSRα2 as before.

Multiple mutations could also be combined by performing the PCR reactions on templates already containing mutations. For example, N39 was made by introducing N36 and N38 changes into N15 template DNA. This was done using a different set of primers (N36(2)) than that used to make N36 (N36(1)). See primers set forth above. Both sets of primers introduced the same mutation.

Longer mpl ligand forms could also be made. Thus, N40 was made in a similar manner to N39 except the 3' reverse primer in PCR 3, (step 1) and the PCR primer in step 2 was the primer used to make N31. This primer introduces a stop codon at position 200 followed by a SalI restriction site. In addition, the template DNA used for PCR 3 contained sequences encoding full length mpl ligand (1-332).

The typical PCR reaction mix contained: 4 ul each of forward and reverse primers (5 pm/ul), 1 ul template (50 ng), 10 ul of 5X LP buffer (100 mM Tricine pH 8.7/25% glycerol/425 mM KOAc), 10 ul dNTP stock (1 mM each of dATP, dTTP, dCTP, dGTP), 0.8 ul rtTh polymerase (Perkin Elmer; 2.5 U/ul), and 2 ul Vent polymerase (NEB; 0.01 U/ul after 1:100 fresh dilution in 1X LP buffer).

$H_2O$ was added to bring the final volume to 50 ul. All the components were added together in the order shown and the PCR was started when the temperature during the first cycle was above the 60°C by adding 1 ul of 50 mM MgOAc. Reaction conditions were: 2 cycles of 94°C, 10 sec/45°C, 1 min/ 68°C, 5 min followed by 25 cycles of 94°C, 10 sec/55°C, 1 min/ 68°C, 5 min.

These general procedures were used to construct the mpl ligand analogs and truncations N16 to N40 shown in Table 6. The DNA sequence changes for each of the forms are shown.

TABLE 6

MPL LIGAND ANALOGS HAVING SITES
FOR N-LINKED CARBOHYDRATE CHAINS

| Analog/ Species No. | Amino Acid Substitution | Sequence Changes |
|---|---|---|
| N16 | (1-183); $Thr^{184} \rightarrow Gly^{332}$ deleted | ACA→TGA (stop codon) |
| N17 | (1-191); $Thr^{192} \rightarrow Gly^{332}$ deleted | ACT→TAA (stop codon) |
| N18 | $His^{23}, Arg^{25} \rightarrow Asn^{23}, Ser^{25}$ | CAC, AGA→AAC, AGC |
| N19 | $Thr^{37}, Pro^{38}, Val^{39} \rightarrow Asn^{37}, Gly^{38}, Ser^{39}$ | ACA, CCT, GTC →AAC, GGT, TCC |
| N20 | $Val^{39}$, $Leu^{41} \rightarrow Asn^{39}$, $Ser^{41}$ | GTC, CTG→AAC, TCG |
| N21 | $Gln^{54}, Glu^{56} \rightarrow Asn^{54}, Ser^{56}$ | CAG, GAG→AAT, TCG |
| N22 | $Lys^{52}$, $Gln^{54} \rightarrow Asn^{52}$, $Thr^{54}$ | AAA, CAG→AAC, ACG |
| N23 | $Glu^{57} \rightarrow Asn^{55'(i)}, Thr^{57}$ | GAG→AAC(i), ACG |
| N24 | $Glu^{57}, Lys^{59} \rightarrow Asn^{57}, Ser^{59}$ | GAG, AAG→AAC, TCG |
| N25 | $Leu^{81}, Pro^{83} \rightarrow Asn^{81}, Thr^{83}$ | CTG, CCC→AAC, ACC |
| N26 | $Thr^{118}, Ala^{120} \rightarrow Asn^{118}, Ser^{120}$ | ACC, GCT→AAC, TCT |
| N27 | $Thr^{119}, His^{121} \rightarrow Asn^{119}, Ser^{121}$ | ACA, CAC→AAC, AGC |
| N29 | $Pro^{30}, Val^{32}, Ala^{120}, Lys^{122} \rightarrow Asn^{30}, Ser^{32}, Asn^{120}, Ser^{122}$ | CCA, GTT, GCT, AAG→ AAC, TCC, AAT, TCC |
| N30 | $Ser^{163}, Arg^{164} \rightarrow Thr^{163}, Asn^{164}$ | AGC, AGA→ACC, AAC |

| | | |
|---|---|---|
| N31 | (1-199): $Trp^{200}\rightarrow Gly^{332}$ deleted | TGG→TGA (stop codon) |
| N33 | $Pro^{30}, Val^{32}, Glu^{57}, Ala^{120}, Lys^{122}$ → $Asn^{30}, Thr^{32}, Asn^{55'}(i), Thr^{57}$ $Asn^{120}, Thr^{122}$ | CCA, GTT, GAG, GCT, AAG → AAC, TCC, AAC(i), ACG, AAT, TCC |
| N34 | $Pro^{30}, Val^{32}, Glu^{57}, Ser^{163}, Arg^{164}$ → $Asn^{30}, Thr^{32}, Asn^{55'(i)}, Thr^{57}$, $Thr^{163}, Asn^{164}$ | CCA, GTT, GAG, AGC, AGA → AAC, TCC, AAC(i), ACG, ACC, AAC |
| N35 | N4+N23+N30+N31 (1-199) | - - |
| N36 | $Met^{55}, Glu^{57}\rightarrow Asn^{55}, Thr^{57}$ | ATG, GAG→AAC, ACC |
| N37 | $Glu^{56}\rightarrow Asn^{56}$ | GAG→AAC |
| N38 | $Ser^{163}, Arg^{164}, Ser^{166}$ $\rightarrow Thr^{163}, Asn^{164}, Thr^{166}$ | AGC, AGA, TCT →ACC, AAC, ACT |
| N39 | N4+N10+N36+N38 (1-174) | - - |
| N40 | N4+N10+N36+N38+N31 (1-199) | - - |

The symbol "(i)" in the above Table means that the referenced amino acid has been inserted. For example, $Glu^{57}$->$Asn^{55'(i)}$, $Thr^{57}$ (analog N23 in Table 6) means that the Glu at position 57 has been replaced with a Thr and, additionally, an Asn has been inserted just after the Met at position 55, and the Asn has been numbered 55' so that subsequent amino acids retain their previously assigned numbers.

Examples that include all changes from previous Examples are indicated by the specific analog

numbers joined by "+" signs. See analogs N35, N39, and N40. The lengths of the amino acid chains of these analogs are indicated parenthetically. Thus, analog N35 contains a combination of all changes made for analogs N4, N23, N30 and N31. The changes indicated for N31 mean that analog N35 is 199 amino acids long. All analogs in Table 6 are 174 amino acids long, except where indicated to be a different length (or, in the cases where an amino acid has been inserted, the total length will be increased by the number of inserted amino acids).

EXAMPLE 15

Characterization of Mpl Ligand Analogs and Truncations N16 to N40

A. Determination of expression level and *in vitro* biological activity of mpl ligand analogs.

Species N16 to N40 were transfected into COS cells using either the electroporation method (Example 5) or the CaPO4 method (Mammalian cell Transfection Kit; Specialty media). Cell free conditioned medium was collected after 3-4 days, aliquoted and stored at -70 °C. Expression level was determined by ELISA assay as described in Example 7. The supernatants were also assayed for biological activity as described in Example 9 with one modification. The activities were calculated from a standard curve using purified CHO cell expressed mpl ligand 1-332 as standard.

The results are shown in Table 7. As shown in Table 7 most of the mpl ligand analogs were expressed and secreted. Some of the analogs appeared to have increased secretion. Bioassays on these samples indicated that the specific activities for most were

also comparable to unmodified forms. Some of the analogs contained multiple N-linked carbohydrate chains(see below). This indicates that carbohydrate addition can result in increased secretion and normal *in vitro* activity of the analogs.

TABLE 7

| Mpl Ligand Form (Amino Acid Length) | Sequence | Number of N-linked chains (a) | Elisa (ng/ml) (b) | In Vitro Activity (units/ml) (c) | Specific Activity (units/ng) (d) |
|---|---|---|---|---|---|
| N1 (174) | Native | 0 | 28 | 3991 | 143 |
| N15 (174) | N30T32N120T122 | 0-2 | 45 | 7003 | 156 |
| N16 (183) | 1-183 | 0 | 85 | 9276 | NA |
| N17 (191) | 1-191 | NA | <0.3 | 11 | NA |
| N18 (174) | N23S25 | 0 | 2 | 5 | 2.5 |
| N19 (174) | N37G38S39 | NA | <0.3 | NA | NA |
| N20 (174) | N39S41 | NA | <0.3 | <10 | NA |
| N21 (174) | N54S56 | 0-1 | 30 | 4380 | 146 |
| N22 (174) | N52T54 | 0-1 | 2 | 856 | 428 |
| N23 (174) | N55'(i)T57 | 1 | 11 | 1059 | 96 |
| N24 (174) | N57S59 | 0 | 5.3 | 458 | 86 |
| N25 (174) | N81T83 | NA | 0.22 | 123 | 559 |
| N26 (174) | N118S120 | NA | 0.9 | 96 | 106 |
| N27 (174) | N119S121 | 0 | 4.5 | 338 | 75 |
| N29 (174) | N30S32N120S122 | 0-2 | 15 | 1627 | 108 |
| N30 (174) | T163N164 | 0-1 | 128 | 15592 | 122 |
| N31 (199) | 1-199 | at least 1 | 156 | 19000 | 122 |
| N33 (174) | 4+10+23 | 3 | 78 | 10057 | 129 |
| N34 (174) | 4+23+30 | at least 2 | 112 | 13536 | 120 |
| N35 (199) | 34 +31 | 4 or more | 172 | 13112 | 76 |
| N36 (174) | N55T57 | 0-1 | 48 | 5808 | 121 |
| N37 (174) | N56 | 1 | 32 | 4504 | 141 |
| N38 (174) | T163N164T166 | 0-1 | 25 | 3904 | 156 |
| N39 (174) | N4+N10+N36+N38 | 3 to 4 | 127 | 17661 | 139 |
| N40 (199) | N4+N10+N36+N38+N31 | at least 5 | 134 | 19735 | 147 |

NOTES

(a) The number of additional N-linked chains was estimated according to the mobility of the analog polypeptides in SDS gels.

(b) Quantities of mpl ligand analogs in COS cell supernatants were determined by EIA.

(c) In vitro activity was determined by measuring proliferation of 32D-MPL cells which are dependent on mpl ligand for growth.

(d) Ratio of *in vitro* activity of mpl ligand analog as measured by proliferation assays to amount of mpl ligand analog measured by mpl ligand ELISA.

i- Insertion

NA Not available

B. Determination of carbohydrate addition.

The analogs shown in Table 6 were tested to see if they added N-linked carbohydrate using the procedures described in Example 6.

Some analogs (N21, N22, N30, N33, and N36) were also tested with a modified procedure. This was necessary because the monoclonal antibody used to develop the Western blot was raised to a peptide including amino acid residues 47 to 62, and some of the analogs described in Table 6 contain substitutions that affected immunoreactivity with this antibody, e.g. N21. Therefore, to analyze these analogs the supernatants were immunoprecipitated using a monoclonal antibody raised in mice to *E. coli* cell expressed mpl ligand

1-163.

Typically 15 ugs of antibody was used to immunoprecipitate 50 ng of mpl ligand analog. Western blots with immunoprecipitated material were performed as described in Example 6 except the immunoprecipitated bands were visualized by incubating the blots with the rabbit anti-mpl ligand polyclonal antibody (typically 1 ug/ml; raised to *E. coli* cell expressed mpl ligand 1-163) and an anti rabbit ECL kit (Amersham). The results of the various experiments are shown in Table 7. Some of the analogs had increased size indicative of the presence of N-linked carbohydrate (N21, N22, N23, N29, N30, N31, N33, N34, N35, N36, N38, N39, and N40). A subset of these analogs had more than 1 N-linked chain, e.g., N29, N33, N34, N35, N39 and N40. These analogs were secreted at normal or higher levels and had *in vitro* biological activity comparable to mpl ligand 1-174. This indicates that multiple functional N-linked glycosylation sites can be introduced into mpl ligand without a deleterious effect on either expression or biological activity.

To demonstrate that multiple oligosaccharide chains can be added to mpl ligand, various analogs expressed in COS cells were analyzed by Western blot as described in Example 6. FIG. 12 shows that the mobility of the analogs decreases with increasing numbers of added N-linked glycosylation sites. Analogs with 4 new sites are shown, N39 and N40. The analogs with the most N-linked sites had the slowest mobility. This result is observed with both 1-174 and 1-199 forms of mpl ligand. This indicates that at least 4 analogs can be combined together resulting in new analogs with multiple N-linked carbohydrate chains.

EXAMPLE 16

Comparison of Glycosylation Sites Containing Asn-X-Ser vs. Asn-X-Thr

N-linked glycosylation sites include either Asn-X-Thr or Asn-X-Ser where X can be any one of the 20 naturally occurring amino acids except Pro. We wished to determine whether Ser or Thr is preferred in the third position. Therefore, two sets of analogs with each set containing a mpl ligand glycosylation analog containing either a Ser or Thr at the third position in the sequon were examined to see if there was an effect on percent occupancy of the N-linked glycosylation sites. N15 contains 2 Asn-X-Thr sites while N29 contains 2 Asn-X-Ser sites at exactly the same positions. In a similar manner N30 contains an Asn-X-Ser while N38 contains an Asn-X-Thr at the same position.

To compare these two sets of analogs, they were expressed in COS cells and the secreted mpl ligand was subjected to Western analysis as described in Example 6. FIG. 13 shows the results. N15 had a significantly increased proportion of glycosylated mpl ligand as compared to N29. In contrast, there was very little difference in the proportion of glycosylated and unglycosylated mpl ligand when N30 and N38 were compared. These results indicate that both Asn-X-Ser and Asn-X-Thr can be introduced into mpl ligand and that both can act as sites for N-linked carbohydrate addition. In addition, in some cases the Asn-X-Thr sequon may be preferred (i.e., it may be more efficiently glycosylated).

While the invention has been described in what is considered to be its preferred embodiments, it is not to be limited to the disclosed embodiments, but on the

contrary, is intended to cover various modifications and equivalents included within the spirit and scope of the appended claims, which scope is to be accorded the broadest interpretation so as to encompass all such modifications and equivalents.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Elliott, Steven G.

(ii) TITLE OF INVENTION: MPL LIGAND ANALOGS

(iii) NUMBER OF SEQUENCES: 56

(iv) CORRESPONDENCE ADDRESS:
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(B) STREET: 1840 Dehavilland Drive
(C) CITY: Thousand Oaks
(D) STATE: CA
(E) COUNTRY: USA
(F) ZIP: 91320-1789

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1342 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 36..1094

(ix) FEATURE:
(A) NAME/KEY: sig_peptide
(B) LOCATION: 1..98

(ix) FEATURE:
(A) NAME/KEY: mat_peptide
(B) LOCATION: 99..1094

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

```
CAGGGAGCCA CGCCAGCCAA GACACCCCGG CCAGA ATG GAG CTG ACT GAA TTG          53
                                       Met Glu Leu Thr Glu Leu
                                       -21 -20

CTC CTC GTG GTC ATG CTT CTC CTA ACT GCA AGG CTA ACG CTG TCC AGC        101
Leu Leu Val Val Met Leu Leu Leu Thr Ala Arg Leu Thr Leu Ser Ser
-15                 -10                 -5                  1

CCG GCT CCT CCT GCT TGT GAC CTC CGA GTC CTC AGT AAA CTG CTT CGT        149
Pro Ala Pro Pro Ala Cys Asp Leu Arg Val Leu Ser Lys Leu Leu Arg
                    5                  10                  15

GAC TCC CAT GTC CTT CAC AGC AGA CTG AGC CAG TGC CCA GAG GTT CAC        197
Asp Ser His Val Leu His Ser Arg Leu Ser Gln Cys Pro Glu Val His
            20                  25                  30

CCT TTG CCT ACA CCT GTC CTG CTG CCT GCT GTG GAC TTT AGC TTG GGA        245
Pro Leu Pro Thr Pro Val Leu Leu Pro Ala Val Asp Phe Ser Leu Gly
        35                  40                  45

GAA TGG AAA ACC CAG ATG GAG GAG ACC AAG GCA CAG GAC ATT CTG GGA        293
Glu Trp Lys Thr Gln Met Glu Glu Thr Lys Ala Gln Asp Ile Leu Gly
    50                  55                  60                  65

GCA GTG ACC CTT CTG CTG GAG GGA GTG ATG GCA GCA CGG GGA CAA CTG        341
Ala Val Thr Leu Leu Leu Glu Gly Val Met Ala Ala Arg Gly Gln Leu
                    70                  75                  80

GGA CCC ACT TGC CTC TCA TCC CTC CTG GGG CAG CTT TCT GGA CAG GTC        389
Gly Pro Thr Cys Leu Ser Ser Leu Leu Gly Gln Leu Ser Gly Gln Val
                85                  90                  95

CGT CTC CTC CTT GGG GCC CTG CAG AGC CTC CTT GGA ACC CAG CTT CCT        437
Arg Leu Leu Leu Gly Ala Leu Gln Ser Leu Leu Gly Thr Gln Leu Pro
            100                 105                 110

CCA CAG GGC AGG ACC ACA GCT CAC AAG GAT CCC AAT GCC ATC TTC CTG        485
Pro Gln Gly Arg Thr Thr Ala His Lys Asp Pro Asn Ala Ile Phe Leu
        115                 120                 125

AGC TTC CAA CAC CTG CTC CGA GGA AAG GTG CGT TTC CTG ATG CTT GTA        533
Ser Phe Gln His Leu Leu Arg Gly Lys Val Arg Phe Leu Met Leu Val
130                 135                 140                 145

GGA GGG TCC ACC CTC TGC GTC AGG CGG GCC CCA CCC ACC ACA GCT GTC        581
Gly Gly Ser Thr Leu Cys Val Arg Arg Ala Pro Pro Thr Thr Ala Val
                150                 155                 160

CCC AGC AGA ACC TCT CTA GTC CTC ACA CTG AAC GAG CTC CCA AAC AGG        629
Pro Ser Arg Thr Ser Leu Val Leu Thr Leu Asn Glu Leu Pro Asn Arg
            165                 170                 175

ACT TCT GGA TTG TTG GAG ACA AAC TTC ACT GCC TCA GCC AGA ACT ACT        677
Thr Ser Gly Leu Leu Glu Thr Asn Phe Thr Ala Ser Ala Arg Thr Thr
        180                 185                 190
```

```
GGC TCT GGG CTT CTG AAG TGG CAG CAG GGA TTC AGA GCC AAG ATT CCT      725
Gly Ser Gly Leu Leu Lys Trp Gln Gln Gly Phe Arg Ala Lys Ile Pro
     195                 200                 205

GGT CTG CTG AAC CAA ACC TCC AGG TCC CTG GAC CAA ATC CCC GGA TAC      773
Gly Leu Leu Asn Gln Thr Ser Arg Ser Leu Asp Gln Ile Pro Gly Tyr
210                 215                 220                 225

CTG AAC AGG ATA CAC GAA CTC TTG AAT GGA ACT CGT GGA CTC TTT CCT      821
Leu Asn Arg Ile His Glu Leu Leu Asn Gly Thr Arg Gly Leu Phe Pro
                    230                 235                 240

GGA CCC TCA CGC AGG ACC CTA GGA GCC CCG GAC ATT TCC TCA GGA ACA      869
Gly Pro Ser Arg Arg Thr Leu Gly Ala Pro Asp Ile Ser Ser Gly Thr
            2                   250                 255

TCA GAC ACA GGC TCC CTG CCA CCC AAC CTC CAG CCT GGA TAT TCT CCT      917
Ser Asp Thr Gly Ser Leu Pro Pro Asn Leu Gln Pro Gly Tyr Ser Pro
          260                 265                 270

TCC CCA ACC CAT CCT CCT ACT GGA CAG TAT ACG CTC TTC CCT CTT CCA      965
Ser Pro Thr His Pro Pro Thr Gly Gln Tyr Thr Leu Phe Pro Leu Pro
     275                 280                 285

CCC ACC TTG CCC ACC CCT GTG GTC CAG CTC CAC CCC CTG CTT CCT GAC     1013
Pro Thr Leu Pro Thr Pro Val Val Gln Leu His Pro Leu Leu Pro Asp
290                 295                 300                 305

CCT TCT GCT CCA ACG CCC ACC CCT ACC AGC CCT CTT CTA AAC ACA TCC     1061
Pro Ser Ala Pro Thr Pro Thr Pro Thr Ser Pro Leu Leu Asn Thr Ser
                    310                 315                 320

TAC ACC CAC TCC CAG AAT CTG TCT CAG GAA GGG TAAGGTTCTC AGACACTGCC    1114
Tyr Thr His Ser Gln Asn Leu Ser Gln Glu Gly
            325                 330

GACATCAGCA TTGTCTCGTG TACAGCTCCC TTCCCTGCAG GGCGCCCCTG GGAGACAACT    1174

GGACAAGATT TCCTACTTTC TCCTGAAACC CAAAGCCCTG GTAAAAGGGA TACACAGGAC    1234

TGAAAAGGGA ATCATTTTTC ACTGTACATT ATAAACCTTC AGAAGCTATT TTTTTAAGCT    1294

ATCAGCAATA CTCATCAGAG CAGCTAGCTC TTTGGTCTAT TTTCTGCA                 1342
```

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 353 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```
Met Glu Leu Thr Glu Leu Leu Leu Val Val Met Leu Leu Leu Thr Ala
-21 -20                 -15                 -10

Arg Leu Thr Leu Ser Ser Pro Ala Pro Pro Ala Cys Asp Leu Arg Val
 5                   1                   5                  10

Leu Ser Lys Leu Leu Arg Asp Ser His Val Leu His Ser Arg Leu Ser
                15                  20                  25

Gln Cys Pro Glu Val His Pro Leu Pro Thr Pro Val Leu Leu Pro Ala
            30                  35                  40

Val Asp Phe Ser Leu Gly Glu Trp Lys Thr Gln Met Glu Glu Thr Lys
        45                  50                  55

Ala Gln Asp Ile Leu Gly Ala Val Thr Leu Leu Leu Glu Gly Val Met
 60                  65                  70                  75

Ala Ala Arg Gly Gln Leu Gly Pro Thr Cys Leu Ser Ser Leu Leu Gly
                80                  85                  90

Gln Leu Ser Gly Gln Val Arg Leu Leu Leu Gly Ala Leu Gln Ser Leu
            95                  100                 105

Leu Gly Thr Gln Leu Pro Pro Gln Gly Arg Thr Thr Ala His Lys Asp
        110                 115                 120

Pro Asn Ala Ile Phe Leu Ser Phe Gln His Leu Leu Arg Gly Lys Val
    125                 130                 135

Arg Phe Leu Met Leu Val Gly Gly Ser Thr Leu Cys Val Arg Arg Ala
140                 145                 150                 155

Pro Pro Thr Thr Ala Val Pro Ser Arg Thr Ser Leu Val Leu Thr Leu
                160                 165                 170

Asn Glu Leu Pro Asn Arg Thr Ser Gly Leu Leu Glu Thr Asn Phe Thr
            175                 180                 185

Ala Ser Ala Arg Thr Thr Gly Ser Gly Leu Leu Lys Trp Gln Gln Gly
        190                 195                 200

Phe Arg Ala Lys Ile Pro Gly Leu Leu Asn Gln Thr Ser Arg Ser Leu
    205                 210                 215

Asp Gln Ile Pro Gly Tyr Leu Asn Arg Ile His Glu Leu Leu Asn Gly
220                 225                 230                 235

Thr Arg Gly Leu Phe Pro Gly Pro Ser Arg Arg Thr Leu Gly Ala Pro
                240                 245                 250

Asp Ile Ser Ser Gly Thr Ser Asp Thr Gly Ser Leu Pro Pro Asn Leu
            255                 260                 265

Gln Pro Gly Tyr Ser Pro Ser Pro Thr His Pro Pro Thr Gly Gln Tyr
        270                 275                 280
```

```
Thr Leu Phe Pro Leu Pro Pro Thr Leu Pro Thr Pro Val Val Gln Leu
     285                 290                 295

His Pro Leu Leu Pro Asp Pro Ser Ala Pro Thr Pro Thr Pro Thr Ser
300                 305                 310                 315

Pro Leu Leu Asn Thr Ser Tyr Thr His Ser Gln Asn Leu Ser Gln Glu
                    320                 325                 330

Gly
```

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 600 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 12..596

(ix) FEATURE:
(A) NAME/KEY: sig_peptide
(B) LOCATION: 12..74

(ix) FEATURE:
(A) NAME/KEY: mat_peptide
(B) LOCATION: 75..96

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

```
TCTAGACCAC C ATG GAG CTG ACT GAA TTG CTC CTC GTG GTC ATG CTT CTC      50
             Met Glu Leu Thr Glu Leu Leu Leu Val Val Met Leu Leu
             -21 -20                 -15                 -10

CTA ACT GCA AGG CTA ACG CTG TCC AGC CCG GCT CCT CCT GCT TGT GAC      98
Leu Thr Ala Arg Leu Thr Leu Ser Ser Pro Ala Pro Pro Ala Cys Asp
               5                   1               5

CTC CGA GTC CTC AGT AAA CTG CTT CGT GAC TCC CAC GTC CTT CAC AGC     146
Leu Arg Val Leu Ser Lys Leu Leu Arg Asp Ser His Val Leu His Ser
      10                  15                  20

AGA CTG AGC CAG TGC CCA GAG GTT CAC CCT TTG CCT ACA CCT GTC CTG     194
Arg Leu Ser Gln Cys Pro Glu Val His Pro Leu Pro Thr Pro Val Leu
 25                  30                  35                  40

CTG CCT GCT GTG GAC TTT AGC TTG GGA GAA TGG AAA ACC CAG ATG GAG     242
Leu Pro Ala Val Asp Phe Ser Leu Gly Glu Trp Lys Thr Gln Met Glu
                    45                  50                  55
```

```
GAG ACC AAG GCA CAG GAC ATT CTG GGA GCA GTG ACC CTT CTG CTG GAG      290
Glu Thr Lys Ala Gln Asp Ile Leu Gly Ala Val Thr Leu Leu Leu Glu
                60                  65                  70

GGA GTG ATG GCA GCA CGG GGA CAA CTG GGA CCC ACT TGC CTC TCA TCC      338
Gly Val Met Ala Ala Arg Gly Gln Leu Gly Pro Thr Cys Leu Ser Ser
            75                  80                  85

CTC CTG GGG CAG CTT TCT GGA CAG GTC CGT CTC CTC CTT GGG GCC CTG      386
Leu Leu Gly Gln Leu Ser Gly Gln Val Arg Leu Leu Leu Gly Ala Leu
        90                  95                  100

CAG AGC CTC CTT GGA ACC CAG CTT CCT CCA CAG GGC AGG ACC ACA GCT      434
Gln Ser Leu Leu Gly Thr Gln Leu Pro Pro Gln Gly Arg Thr Thr Ala
105                 110                 115                 120

CAC AAG GAT CCC AAT GCC ATC TTC CTG AGC TTC CAA CAC CTG CTC CGA      482
His Lys Asp Pro Asn Ala Ile Phe Leu Ser Phe Gln His Leu Leu Arg
                125                 130                 135

GGA AAG GTG CGT TTC CTG ATG CTT GTA GGA GGG TCC ACC CTC TGC GTC      530
Gly Lys Val Arg Phe Leu Met Leu Val Gly Gly Ser Thr Leu Cys Val
            140                 145                 150

AGG CGG GCC CCA CCC ACC ACA GCT GTC CCC AGC AGA ACC TCT CTA GTC      578
Arg Arg Ala Pro Pro Thr Thr Ala Val Pro Ser Arg Thr Ser Leu Val
        155                 160                 165

CTC ACA CTG AAC GAG CTC TAGG                                        600
Leu Thr Leu Asn Glu Leu
    170
```

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 195 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

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Met Glu Leu Thr Glu Leu Leu Leu Val Val Met Leu Leu Leu Thr Ala
-21 -20                 -15                 -10

Arg Leu Thr Leu Ser Ser Pro Ala Pro Pro Ala Cys Asp Leu Arg Val
 5                   1                   5                  10

Leu Ser Lys Leu Leu Arg Asp Ser His Val Leu His Ser Arg Leu Ser
                15                  20                  25

Gln Cys Pro Glu Val His Pro Leu Pro Thr Pro Val Leu Leu Pro Ala
            30                  35                  40

Val Asp Phe Ser Leu Gly Glu Trp Lys Thr Gln Met Glu Glu Thr Lys
        45                  50                  55
```

Ala Gln Asp Ile Leu Gly Ala Val Thr Leu Leu Leu Glu Gly Val Met
60 65 70 75

Ala Ala Arg Gly Gln Leu Gly Pro Thr Cys Leu Ser Ser Leu Leu Gly
80 85 90

Gln Leu Ser Gly Gln Val Arg Leu Leu Leu Gly Ala Leu Gln Ser Leu
95 100 105

Leu Gly Thr Gln Leu Pro Pro Gln Gly Arg Thr Thr Ala His Lys Asp
110 115 120

Pro Asn Ala Ile Phe Leu Ser Phe Gln His Leu Leu Arg Gly Lys Val
125 130 135

Arg Phe Leu Met Leu Val Gly Gly Ser Thr Leu Cys Val Arg Arg Ala
140 145 150 155

Pro Pro Thr Thr Ala Val Pro Ser Arg Thr Ser Leu Val Leu Thr Leu
160 165 170

Asn Glu Leu

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "nucleic acid"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CCCATGTCAA TCACAGCAGA CT 22

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "nucleic acid"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CTTCACAGCA ACCTGAGCCA GT 22

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "nucleic acid"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

CAGTGCAACG AGACCCACCC TTTG 24

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "nucleic acid"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GCCTACAAAT GTCACGCTGC CTGCT 25

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "nucleic acid"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

CCCACTTGTA ACTCATCCCT C 21

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "nucleic acid"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

CAACTGAACG CCACTTGTCT CTCA 24

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "nucleic acid"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

ACTTGTCTCA ACTCCACCCT GGGGGA 26

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "nucleic acid"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

CTCCTGGGGA ACCTTTCTGG A 21

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "nucleic acid"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

GACCACAAAT CACACCGATC CCAAT 25

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "nucleic acid"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

ACCCTTTGTC TACAAATGTC ACGCTGCCTG CT 32

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "nucleic acid"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

TCTCTCAAAC CTCACGGGGG AGCTT 25

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "nucleic acid"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

TGGAAAAATC AGACGGAGGA GAC 23

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "nucleic acid"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

TGGAGGAGAA CAAGACACAG GACAT 25

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 38 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "nucleic acid"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

CCCTCTAGAC CACCATGGAA CTGACTGAAT TGCTCCTC 38

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "nucleic acid"

(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION: complement (1..28)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

GTGTGACTTG CTCGAGACTC AGCTGCCC 28

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "nucleic acid"

(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION: complement (1..29)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

GAAGACCTAA CAACCTCACT CAGCTGCCC 29

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "nucleic acid"

(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION: complement (1..29)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

AGTGACGGAG TCGGTCTATT CAGCTGCCC 29

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "nucleic acid"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

CACGTCCTTA ACAGCAGCCT GAGCCAGTG 29

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "nucleic acid"

(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION: complement (1..29)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

GTGCAGGAAT TGTCGTCGGA CTCGGTCAC 29

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "nucleic acid"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

CCCTTTGCCT AACGGTTCCC TGCTGCCTGC TGT 33

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "nucleic acid"

(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION: complement (1..33)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

GGGAAACGGA TTGCCAAGGG ACGACGGACG ACA 33

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "nucleic acid"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

TGCCTACACC TAACCTGTCG CCTGCTGTGG A 31

(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "nucleic acid"

(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION: complement (1..31)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

ACGGATGTGG ATTGGACAGC GGACGACACC T 31

(2) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "nucleic acid"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

GGAAAACCAA TATGTCGGAG ACCAAGGCAC A 31

(2) INFORMATION FOR SEQ ID NO:29:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "nucleic acid"

(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION: complement (1..31)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

CCTTTTGGTT ATACAGCCTC TGGTTCCGTG T 31

(2) INFORMATION FOR SEQ ID NO:30:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "nucleic acid"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

TGGGAGAATG GAACACCACG ATGGAGGAGA CC 32

(2) INFORMATION FOR SEQ ID NO:31:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "nucleic acid"

(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION: complement (1..32)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

ACCCTCTTAC CTTGTGGTGC TACCTCCTCT GG 32

(2) INFORMATION FOR SEQ ID NO:32:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "nucleic acid"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

AAAACCCAGA TGAACGAGAC GACCAAGGCA CA 32

(2) INFORMATION FOR SEQ ID NO:33:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "nucleic acid"

(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION: complement (1..32)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

TTTTGGGTCT ACTTGCTCTG CTGGTTCCGT GT 32

(2) INFORMATION FOR SEQ ID NO:34:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "nucleic acid"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

CCCAGATGGA GAACACCTCG GCACAGGACA T 31

(2) INFORMATION FOR SEQ ID NO:35:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "nucleic acid"

(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION: complement (1..31)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

GGGTCTACCT CTTGTGGAGC CGTGTCCTGT A 31

(2) INFORMATION FOR SEQ ID NO:36:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "nucleic acid"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:36:

CACGGGGACA AAACGGAACC ACTTGCCTCT CA 32

(2) INFORMATION FOR SEQ ID NO:37:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "nucleic acid"

(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION: complement (1..32)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:37:

GTGCCCCTGT TTTGCCTTGG TGAACGGAGA GT 32

(2) INFORMATION FOR SEQ ID NO:38:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "nucleic acid"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:38:

CAGGGCAGGA ACACATCTCA CAAGGATCCC A 31

(2) INFORMATION FOR SEQ ID NO:39:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "nucleic acid"

(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION: complement (1..31)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:39:

GTCCCGTCCT TGTGTAGAGT GTTCCTAGGG T 31

(2) INFORMATION FOR SEQ ID NO:40:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "nucleic acid"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:40:

GGGCAGGACC AACGCTAGCA AGGATCCCAA T 31

(2) INFORMATION FOR SEQ ID NO:41:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "nucleic acid"

(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION: complement (1..31)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:41:

CCCGTCCTGG TTGCGATCGT TCCTAGGGTT A 31

(2) INFORMATION FOR SEQ ID NO:42:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "nucleic acid"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:42:

CAGTGCAACG AGTCCCACCC TTGG 24

(2) INFORMATION FOR SEQ ID NO:43:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "nucleic acid"

(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION: complement (1..24)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:43:

GTCACGTTGC TCAGGGTGGG AAAC 24

(2) INFORMATION FOR SEQ ID NO:44:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "nucleic acid"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:44:

GACCACAAAT CACTCCGATC CCAA 24

(2) INFORMATION FOR SEQ ID NO:45:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "nucleic acid"

(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION: complement (1..24)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:45:

CTGGTGTTTA GTGAGGCTAG GGTT 24

(2) INFORMATION FOR SEQ ID NO:46:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "nucleic acid"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:46:

GTCCCCACCA ACACCTCTCT AGTCCTC 27

(2) INFORMATION FOR SEQ ID NO:47:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "nucleic acid"

(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION: complement (1..27)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:47:

CAGGGGTGGT TGTGGAGAGA TCAGGAG 27

(2) INFORMATION FOR SEQ ID NO:48:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "nucleic acid"

(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION: complement (1..33)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:48:

TGACCGAGAC CCGAAGACTT CACTCAGCTG CCC 33

(2) INFORMATION FOR SEQ ID NO:49:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs

(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "nucleic acid"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:49:

GAAAACCCAG AACGAGACCA CCAAGGCACA G 31

(2) INFORMATION FOR SEQ ID NO:50:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "nucleic acid"

(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION: complement (1..31)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:50:

CTTTTGGGTC TTGCTCTGGT GGTTCCGTGT C 31

(2) INFORMATION FOR SEQ ID NO:51:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "nucleic acid"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:51:

CACCAAGGCA CAGGACATTC TGGGAG 26

(2) INFORMATION FOR SEQ ID NO:52:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "nucleic acid"

(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION: complement (1..26)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:52:

GTGGTTCCGT GTCCTGTAAG ACCCTC 26

(2) INFORMATION FOR SEQ ID NO:53:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "nucleic acid"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:53:

GAAAACCCAG ATGAACGAGA CCAAGGCACA G 31

(2) INFORMATION FOR SEQ ID NO:54:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "nucleic acid"

(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION: complement (1..31)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:54:

CTTTTGGGTC TACTTGCTCT GGTTCCGTGT C 31

(2) INFORMATION FOR SEQ ID NO:55:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "nucleic acid"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:55:

GTCCCCACCA ACACCACTCT AGTCCTC 27

(2) INFORMATION FOR SEQ ID NO:56:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "nucleic acid"

(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION: complement (1..27)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:56:

CAGGGGTGGT TGTGGTGAGA TCAGGAG 27

WHAT IS CLAIMED IS:

1. An analog of mpl ligand comprising an amino acid sequence which includes at least one added, at least one deleted or at least one altered glycosylation site.

2. The analog of Claim 1 wherein the glycosylation site is for an N-linked carbohydrate chain.

3. The analog of Claim 1 wherein the glycosylation site is for an O-linked carbohydrate chain.

4. The analog of Claim 1 having at least one additional carbohydrate chain attached thereto as expressed in a eukaryote.

5. The analog of Claim 4 wherein the carbohydrate chain is an N-linked carbohydrate chain.

6. The analog of Claim 4 wherein the carbohydrate chain is an O-linked carbohydrate chain.

7. The analog of Claim 1 which is the product of expression of an exogenous DNA sequence in a eukaryote.

8. The analog of Claim 5 which is selected from the group consisting of

[$Asn^{30}$, $Thr^{32}$] mpl ligand;
[$Asn^{82}$, $Ala^{83}$] mpl ligand;
[$Asn^{87}$, $Thr^{89}$] mpl ligand;
[$Asn^{53}$, $Thr^{55}$] mpl ligand;

[Asn$^{58}$, Thr$^{60}$] mpl ligand;
[Asn$^{30}$, Thr$^{32}$, Asn$^{120}$, Thr$^{122}$] mpl ligand;
[Asn$^{37}$, Gly$^{38}$, Ser$^{39}$] mpl ligand;
[Asn$^{39}$, Ser$^{41}$] mpl ligand;
[Asn$^{54}$, Ser$^{56}$] mpl ligand;
[Asn$^{52}$, Thr$^{54}$] mpl ligand;
[Asn$^{55'(i)}$, Thr$^{57}$] mpl ligand;
[Asn$^{81}$, Thr$^{83}$] mpl ligand;
[Asn$^{118}$, Ser$^{120}$] mpl ligand;
[Asn$^{30}$, Ser$^{32}$, Asn$^{120}$, Ser$^{122}$] mpl ligand;
[Thr$^{163}$, Asn$^{164}$] mpl ligand;
[Asn$^{30}$, Thr$^{32}$, Asn$^{120}$, Thr$^{122}$, Asn$^{55(i)}$, Thr$^{57}$] mpl ligand;
[Asn$^{30}$, Thr$^{32}$, Asn$^{55'(i)}$, Thr$^{57}$, Thr$^{163}$, Asn$^{164}$] mpl ligand;
[Asn$^{55}$, Thr$^{57}$] mpl ligand;
[Asn$^{56}$] mpl ligand;
[Thr$^{163}$, Asn$^{164}$, Thr$^{166}$] mpl ligand; and
[Asn$^{30}$, Thr$^{32}$, Asn$^{120}$, Thr$^{122}$, Asn$^{55}$, Thr$^{57}$, Thr$^{163}$, Asn$^{164}$, Thr$^{166}$] mpl ligand.

9. An analog of Claim 1, wherein the mpl ligand has an amino acid sequence selected from the group consisting of:

| | |
|---|---|
| mpl ligand 1-332 | amino acids 1-332 of FIG. 1 |
| mpl ligand 1-199 | amino acids 1-199 of FIG. 1 |
| mpl ligand 1-191 | amino acids 1-191 of FIG. 1 |
| mpl ligand 1-183 | amino acids 1-183 of FIG. 1 |
| mpl ligand 1-174 | amino acids 1-174 of FIG. 1 |
| mpl ligand 1-163 | amino acids 1-163 of FIG. 1 |
| mpl ligand 1-153 | amino acids 1-153 of FIG. 1 |
| mpl ligand 1-152 | amino acids 1-152 of FIG. 1 |
| mpl ligand 1-151 | amino acids 1-151 of FIG. 1 |
| mpl ligand 7-332 | amino acids 7-332 of FIG. 1 |
| mpl ligand 7-191 | amino acids 7-191 of FIG. 1 |
| mpl ligand 7-199 | amino acids 7-199 of FIG. 1 |
| mpl ligand 7-183 | amino acids 7-183 of FIG. 1 |
| mpl ligand 7-174 | amino acids 7-174 of FIG. 1 |

| | |
|---|---|
| mpl ligand 7-163 | amino acids 7-163 of FIG. 1 |
| mpl ligand 7-153 | amino acids 7-153 of FIG. 1 |
| mpl ligand 7-152 | amino acids 7-152 of FIG. 1 |
| mpl ligand 7-151 | amino acids 7-151 of FIG. 1 |

10. An analog of human mpl ligand selected from the group consisting of:

[$Asn^{22}$] mpl ligand;
[$Asn^{25}$] mpl ligand;
[$Asn^{38}$, $Thr^{40}$] mpl ligand;
[$Asn^{86}$] mpl ligand;
[$Asn^{92}$] mpl ligand;
[$Asn^{120}$, $Thr^{122}$] mpl ligand;
[$Ser^{36}$, $Asn^{38}$, $Thr^{40}$] mpl ligand;
[$Asn^{88}$, $Thr^{90}$] mpl ligand;
[$Asn^{23}$, $Ser^{25}$] mpl ligand;
[$Asn^{57}$, $Ser^{59}$] mpl ligand; and
[$Asn^{119}$, $Ser^{121}$] mpl ligand.

11. An analog of human mpl ligand according to Claim 10, wherein said mpl ligand is selected from the group consisting of:

| | |
|---|---|
| mpl ligand 1-332 | amino acids 1-332 of FIG. 1 |
| mpl ligand 1-199 | amino acids 1-199 of FIG. 1 |
| mpl ligand 1-191 | amino acids 1-191 of FIG. 1 |
| mpl ligand 1-183 | amino acids 1-183 of FIG. 1 |
| mpl ligand 1-174 | amino acids 1-174 of FIG. 1 |
| mpl ligand 1-163 | amino acids 1-163 of FIG. 1 |
| mpl ligand 1-153 | amino acids 1-153 of FIG. 1 |
| mpl ligand 1-152 | amino acids 1-152 of FIG. 1 |
| mpl ligand 1-151 | amino acids 1-151 of FIG. 1 |
| mpl ligand 7-332 | amino acids 7-332 of FIG. 1 |
| mpl ligand 7-199 | amino acids 7-199 of FIG. 1 |
| mpl ligand 7-191 | amino acids 7-191 of FIG. 1 |

| | |
|---|---|
| mpl ligand 7-183 | amino acids 7-183 of FIG. 1 |
| mpl ligand 7-174 | amino acids 7-174 of FIG. 1 |
| mpl ligand 7-163 | amino acids 7-163 of FIG. 1 |
| mpl ligand 7-153 | amino acids 7-153 of FIG. 1 |
| mpl ligand 7-152 | amino acids 7-152 of FIG. 1 |
| mpl ligand 7-151 | amino acids 7-151 of FIG. 1 |

12. An analog according to any of Claims 8-11, which is the product of expression of an exogenous DNA sequence in a eukaryotic cell.

13. An analog according to Claim 12, wherein said eukaryotic cell is a mammalian cell.

14. An analog of Claim 1, which is [$Asn^{30}$, $Thr^{32}$, $Asn^{120}$, $Thr^{122}$, $Asn^{55'(i)}$, $Thr^{57}$, $Thr^{163}$, $Asn^{164}$, $Thr^{166}$] mpl ligand 1-174; or [$Asn^{30}$, $Thr^{32}$, $Asn^{120}$, $Thr^{122}$, $Asn^{55'(i)}$, $Thr^{57}$, $Thr^{163}$, $Asn^{164}$, $Thr^{166}$] mpl ligand 1-199, which is the product of expression of an exogenous DNA sequence in a eukaryotic cell.

15. An analog according to Claim 14, wherein the eukaryotic cell is mammalian.

16. An analog according to Claim 15, wherein said mammalian cell is CHO.

17. An analog of mpl ligand comprising an amino acid sequence which comprises (a) substitution of any of the O-linked carbohydrate sites in mpl ligand with an N-linked carbohydrate site; (b) substitution of any of the N-linked carbohydrate sites in mpl ligand with an O-linked carbohydrate site; (c) substitution of any of the O-linked carbohydrate sites in mpl ligand with a different O-linked carbohydrate site; or (d)

substitution of any of the N-linked carbohydrate sites in mpl ligand with a different N-linked carbohydrate site.

18. A DNA sequence encoding an analog of mpl ligand which includes at least one added, at least one deleted or at least one altered glycosylation site.

19. A DNA sequence encoding an analog of mpl ligand according to any of Claims 1-11 and 13-17.

20. A eukaryotic host cell transfected with a DNA sequence according to Claim 19 in a manner allowing the host cell to express an analog of mpl ligand.

21. A composition comprising a therapeutically effective amount of an mpl ligand analog according to any of Claims 1-11 and 13-17 together with a pharmaceutically acceptable diluent, adjuvant or carrier.

SUBSTITUTE SHEET (RULE 26)

```
  1    CAGGGAGCCACGCCAGCCAAGACACCCCGGCCAGAATGGAGCTGACTGAATTGCTCCTC   53
-21                                        MetGluLeuThrGluLeuLeuLeu  -14

 70   GTGGTCATGCTTCTCCTAACTGCAAGGCTAACGCTGTCCAGCCCGGCTCCTCCTGCTTGT  119
-13   ValValMetLeuLeuLeuThrAlaArgLeuThrLeuSerSerProAlaProProAlaCys    7

120   GACCTCCGAGTCCTCAGTAAACTGCTTCGTGACTCCCATGTCCTTCACAGCAGACTGAGC  179
  8  AspLeuArgValLeuSerLysLeuLeuArgAspSerHisValLeuHisSerArgLeuSer   27

180   CAGTGCCCAGAGGTTCACCCTTTGCCTACACCTGTCCTGCTGCCTGCTGTGGACTTTAGC  239
 28   GlnCysProGluValHisProLeuProThrProValLeuLeuProAlaValAspPheSer   47

240   TTGGGAGAATGGAAAACCCAGATGGAGGAGACCAAGGCACAGGACATTCTGGGAGCAGTG  299
 48   LeuGlyGluTrpLysThrGlnMetGluGluThrLysAlaGlnAspIleLeuGlyAlaVal   67

300   ACCCTTCTGCTGGAGGGAGTGATGGCAGCACGGGGACAACTGGGACCCACTTGCCTCTCA  359
 68   ThrLeuLeuLeuGluGlyValMetAlaAlaArgGlyGlnLeuGlyProThrCysLeuSer   87

360   TCCCTCCTGGGGCAGCTTTCTGGACAGGTCCGTCTCCTCCTTGGGGCCCTGCAGAGCCTC  419
 88   SerLeuLeuGlyGlnLeuSerGlyGlnValArgLeuLeuLeuGlyAlaLeuGlnSerLeu  107
```

FIG. 1A

```
420  CTTGGAACCCAGCTTCCTCACAGGGCAGGACCACAGCTCACAAGGATCCCAATGCCATCC  479
108  LeuGlyThrGlnLeuProProGlnGlyArgThrThrAlaHisLysAspProAsnAlaIle  127

480  TTCCTGAGCTTCCAACACCTGCTCCGAGGAAAGGTGCGTTTCCTGATGCTTGTAGGAGGG  539
128  PheLeuSerPheGlnHisLeuLeuArgGlyLysValArgPheLeuMetLeuValGlyGly  147

540  TCCACCCTCTGCGTCAGGCGGGCCCCACCCACCACAGCTGTCCCCAGCAGAACCTCTCTA  599
148  SerThrLeuCysValArgArgAlaProProThrThrAlaValProSerArgThrSerLeu  167

600  GTCCTCACACTGAACGAGCTCCCAAACAGGACTTCTGGATTGTTGGAGACAAACTTCACT  659
168  ValLeuThrLeuAsnGluLeuProAsnArgThrSerGlyLeuLeuGluThrAsnPheThr  187

660  GCCTCAGCCAGAACTACTGGCTCTGGGCTTCTGAAGTGGCAGCAGGGATTCAGAGCCAAG  719
188  AlaSerAlaArgThrThrGlySerGlyLeuLeuLysTrpGlnGlnGlyPheArgAlaLys  207

720  ATTCCTGGTCTGCTGAACCAAACCTCCAGGTCCCTGGACCAAATCCCCGGATACCTGAAC  779
208  IleProGlyLeuLeuAsnGlnThrSerArgSerLeuAspGlnIleProGlyTyrLeuAsn  227

780  AGGATACACGAACTCTTGAATGGAACTCGTGGACTCTTTCCTGGACCCTCACGCAGGACC  839
228  ArgIleHisGluLeuLeuAsnGlyThrArgGlyLeuPheProGlyProSerArgArgThr  247
```

FIG. 1B

```
 840    CTAGGAGCCCCGGACATTTCCTCAGGAACATCAGACACAGGCTCCCTGCCACCCAACCTC   899
 248    LeuGlyAlaProAspIleSerSerGlyThrSerAspThrGlySerLeuProProAsnLeu   267

 900    CAGCCTGGATATTCTCCTTCCCCAACCCATCCTCCTACTGGACAGTATACGCTCTTCCCT   959
 268    GlnProGlyTyrSerProSerProThrHisProProThrGlyGlnTyrThrLeuPhePro   287

 960    CTTCCACCCACCTTGCCCACCCCTGTGGTCCAGCTCCACCCCCTGCTTCCTGACCCTTCT  1019
 288    LeuProProThrLeuProThrProValValGlnLeuHisProLeuLeuProAspProSer   307

1020    GCTCCAACGCCCACCCCTACCAGCCCTCTTCTAAACACATCCTACACCCACTCCCAGAAT  1079
 308    AlaProThrProThrProThrSerProLeuLeuAsnThrSerTyrThrHisSerGlnAsn   327

1080    CTGTCTCAGGAAGGGTAAGGTTCTCAGACACTGCCGACATCAGCATTGTCTCGTGTACAG  1139
 329    LeuSerGlnGluGlyEnd
1140    CTCCCTTCCCTGCAGGGCGCCCCTGGGAGACAACTGGACAAGATTTCCTACTTTCTCCTG  1199
1200    AAACCCAAAGCCCTGGTAAAAGGGATACACAGGACTGAAAAGGGAATCATTTTTCACTGT  1259
1260    ACATTATAAACCTTCAGAAGCTATTTTTTTAAGCTATCAGCAATACTCATCAGAGCAGCT  1319
1320    AGCTCTTTGGTCTATTTTCTGCA     1342
```

FIG.1C

```
        10                  30                  50
        .   .   .   .   .   .   .   .   .   .   .   .
TCTAGACCACCATGGAGCTGACTGAATTGCTCCTCGTGGTCATGCTTCTCCTAACTGCAA
          M   E   L   T   E   L   L   L   V   V   M   L   L   L   T   A   R

        70                  90                  110
        .   .   .   .   .   .   .   .   .   .   .   .
GGCTAACGCTGTCCAGCCCGGCTCCTCCTGCTTGTGACCTCCGAGTCCTCAGTAAACTGC
  L   T   L   S   S   P   A   P   P   A   C   D   L   R   V   L   S   K   L   L

        130                 150                 170
        .   .   .   .   .   .   .   .   .   .   .   .
TTCGTGACTCCCACGTCCTTCACAGCAGACTGAGCCAGTGCCCAGAGGTTCACCCTTTGC
  R   D   S   H   V   L   H   S   R   L   S   Q   C   P   E   V   H   P   L   P

        190                 210                 230
        .   .   .   .   .   .   .   .   .   .   .   .
CTACACCTGTCCTGCTGCCTGCTGTGGACTTTAGCTTGGGAGAATGGAAAACCCAGATGG
  T   P   V   L   L   P   A   V   D   F   S   L   G   E   W   K   T   Q   M   E

        250                 270                 290
        .   .   .   .   .   .   .   .   .   .   .   .
AGGAGACCAAGGCACAGGACATTCTGGGAGCAGTGACCCTTCTGCTGGAGGGAGTGATGG
  E   T   K   A   Q   D   I   L   G   A   V   T   L   L   L   E   G   V   M   A
```

FIG.2A

SUBSTITUTE SHEET (RULE 26)

```
          310                 330                 350
CAGCACGGGGACAACTGGGACCCACTTGCCTCTCATCCCTCCTGGGGCAGCTTTCTGGAC
  A  R  G  Q  L  G  P  T  C  L  S  S  L  L  G  Q  L  S  G  Q

          370                 390                 410
AGGTCCGTCTCCTCCTTGGGGCCCTGCAGAGCCTCCTTGGAACCCAGCTTCCTCCACAGG
  V  R  L  L  L  G  A  L  Q  S  L  L  G  T  Q  L  P  P  Q  G

          430                 450                 470
GCAGGACCACAGCTCACAAGGATCCCAATGCCATCTTCCTGAGCTTCCAACACCTGCTCC
  R  T  T  A  H  K  D  P  N  A  I  F  L  S  F  Q  H  L  L  R

          490                 510                 530
GAGGAAAGGTGCGTTTCCTGATGCTTGTAGGAGGGTCCACCCTCTGCGTCAGGCGGGCCC
  G  K  V  R  F  L  M  L  V  G  G  S  T  L  C  V  R  R  A  P

          550                 570                 590
CACCCACCACAGCTGTCCCCAGCAGAACCTCTCTAGTCCTCACACTGAACGAGCTCTAGG
  P  T  T  A  V  P  S  R  T  S  L  V  L  T  L  N  E  L  *

TCGAC
```

FIG.2B

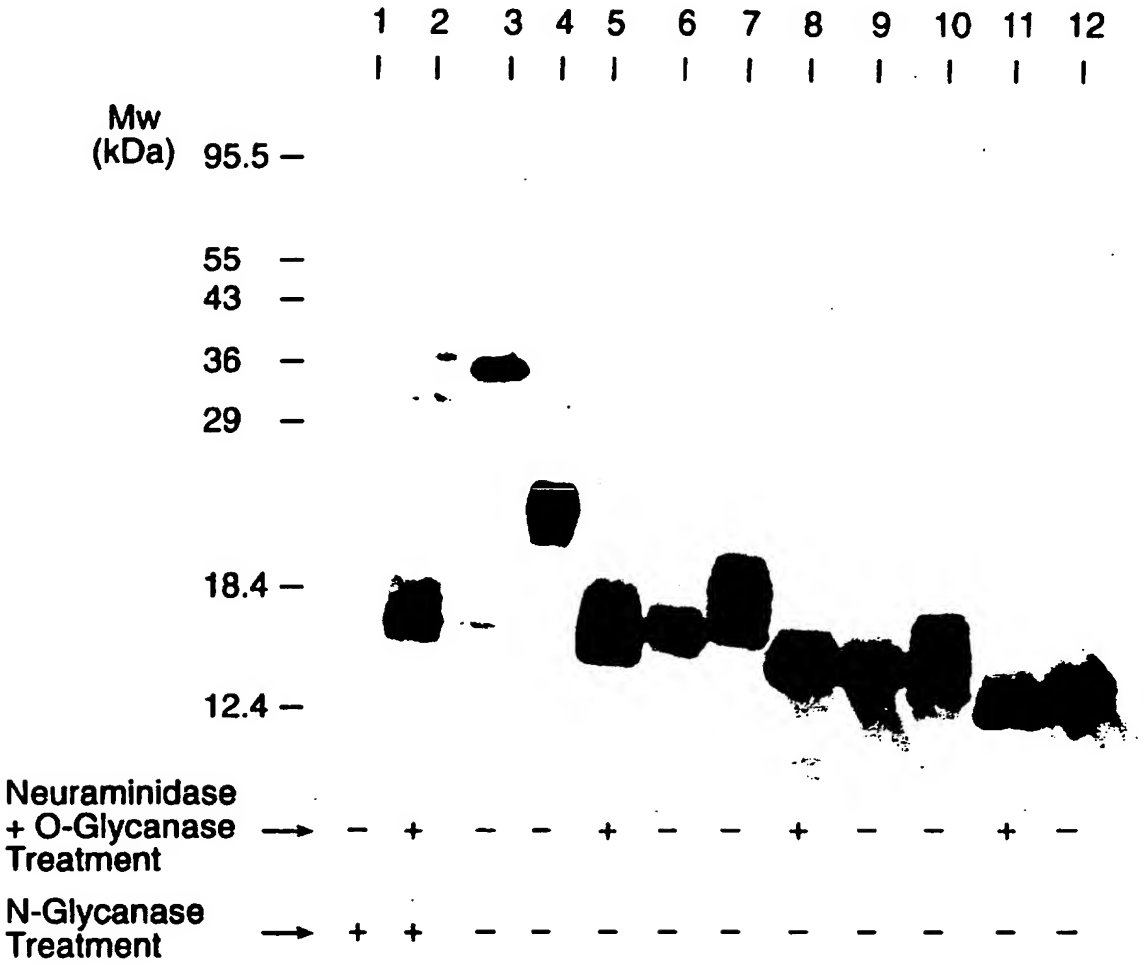
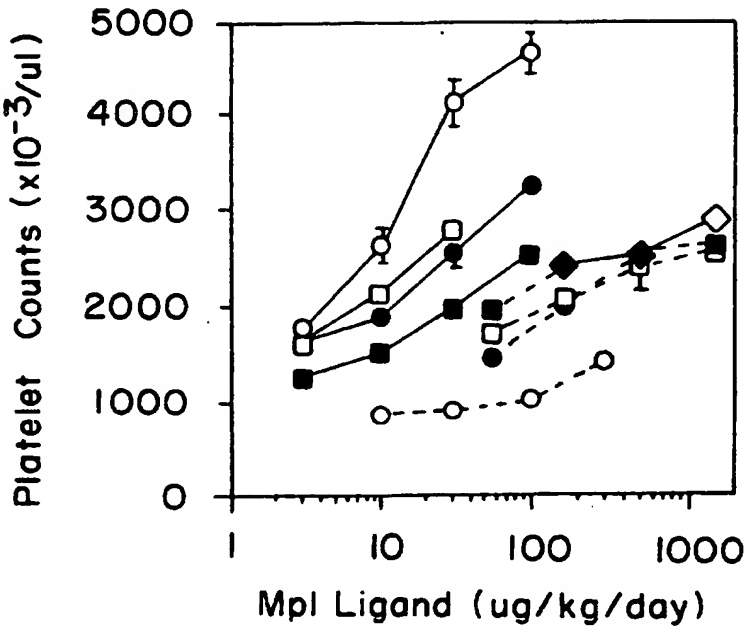

1 2 3 4 5 6 7 8 9 10 11 12
Mw (kDa)
95.5
55
43
36
29
18.4
12.4
Neuraminidase + O-Glycanase Treatment
N-Glycanase Treatment
1. MGDF 1-332 (CHO)
2. MGDF 1-332 (CHO)
3. MGDF 1-332 (E. coli; MK)
4. MGDF 1-174 (CHO)
5. MGDF 1-174 (CHO)
6. MGDF 1-174 (E. coli; MK)
7. MGDF 1-163 (CHO)
8. MGDF 1-163 (CHO)
9. MGDF 1-163 (E. coli; desMK)
10. MGDF 1-153 (CHO)
11. MGDF 1-153 (CHO)
12. MGDF 1-153 (E. coli; MK)

FIG.3

FIG. 4




CHO 332
CHO 174
CHO 163
CHO 153
(coli) MK 332
(coli) MK 174
(coli) MK 163
(coli) MK 153
(coli) desMK 163

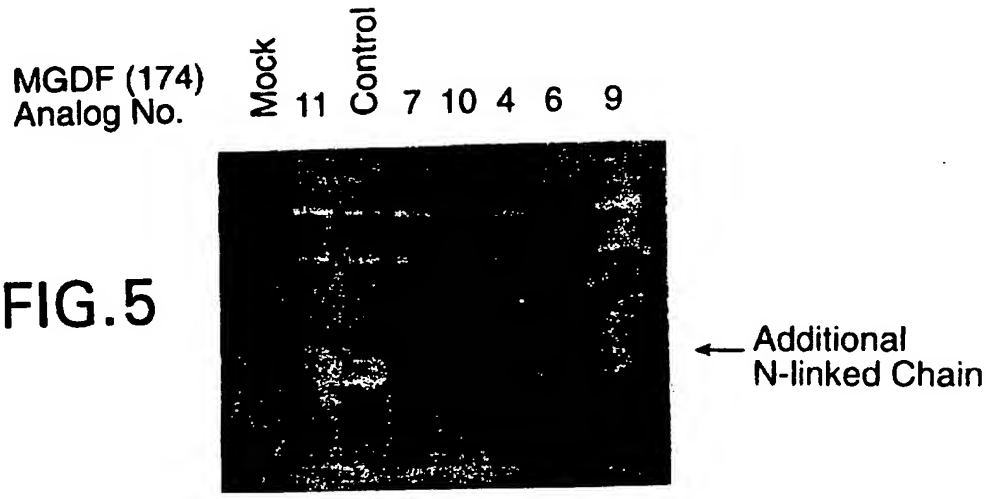



FIG.5

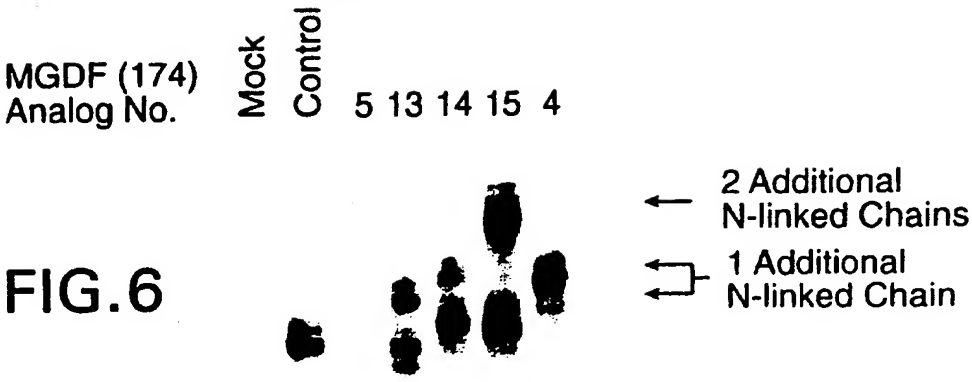



FIG.6

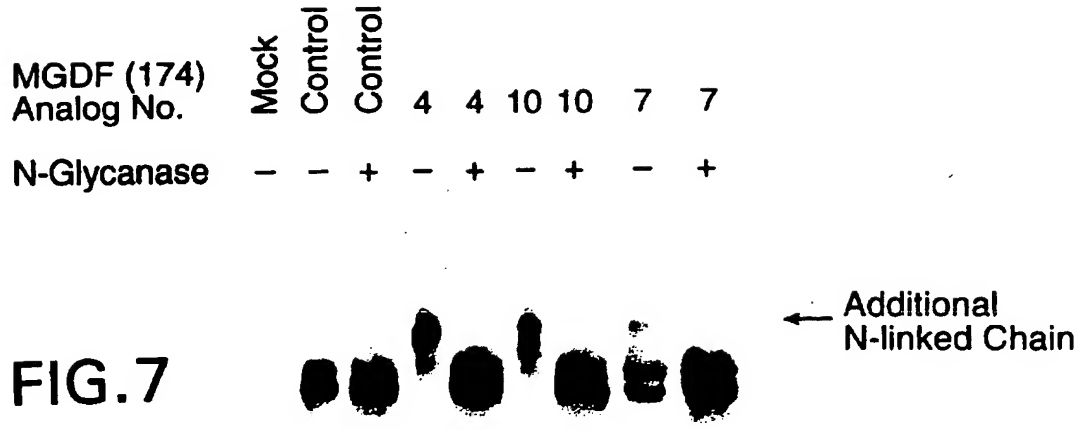



FIG.7

FIG.8A

FIG.8B

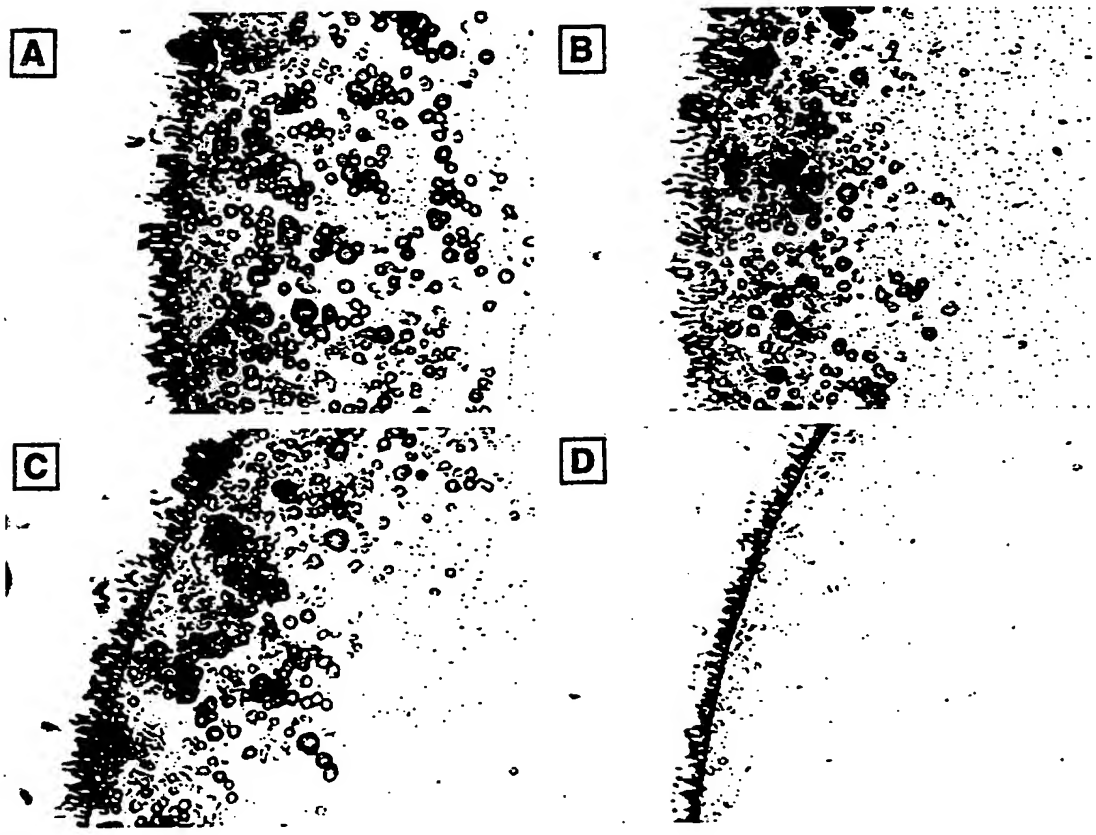



FIG.8C

FIG.8D

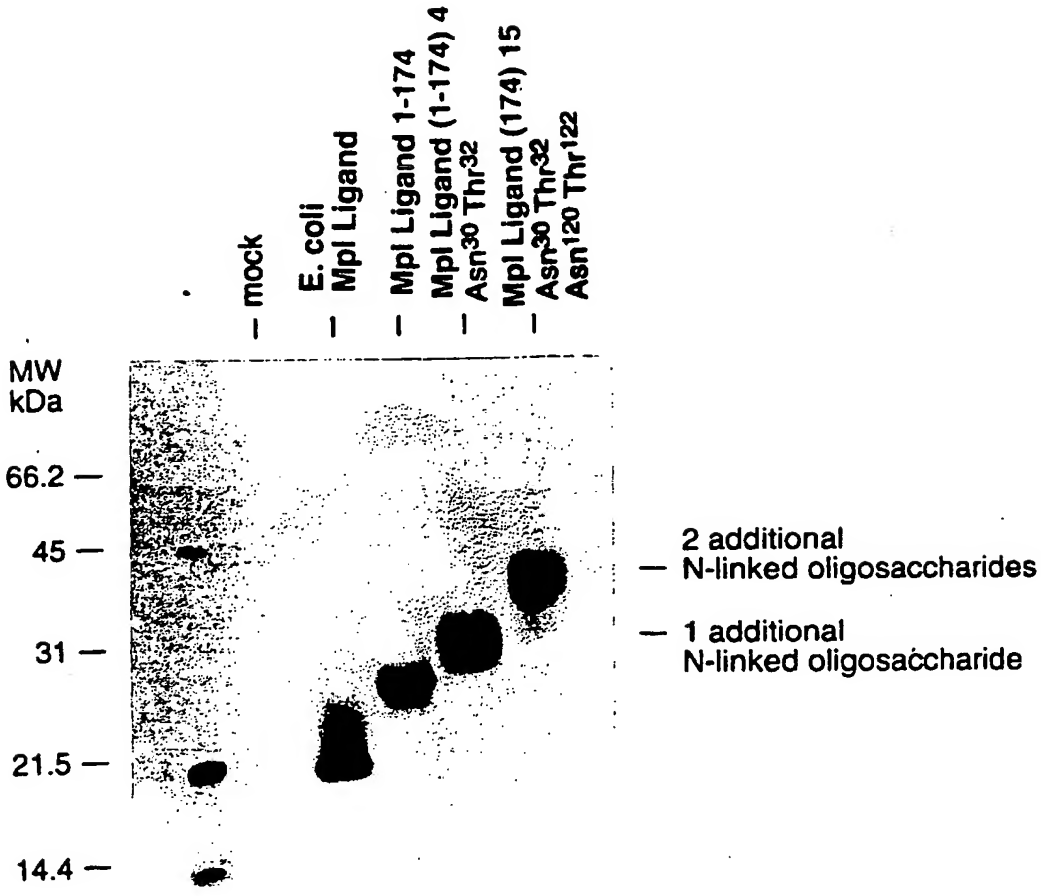

mock
E. coli
Mpl Ligand
Mpl Ligand 1-174
Mpl Ligand (1-174) 4
Asn30 Thr32
Mpl Ligand (174) 15
Asn30 Thr32
Asn120 Thr122
MW
kDa
66.2
45
31
21.5
14.4
2 additional
N-linked oligosaccharides
1 additional
N-linked oligosaccharide

FIG.9

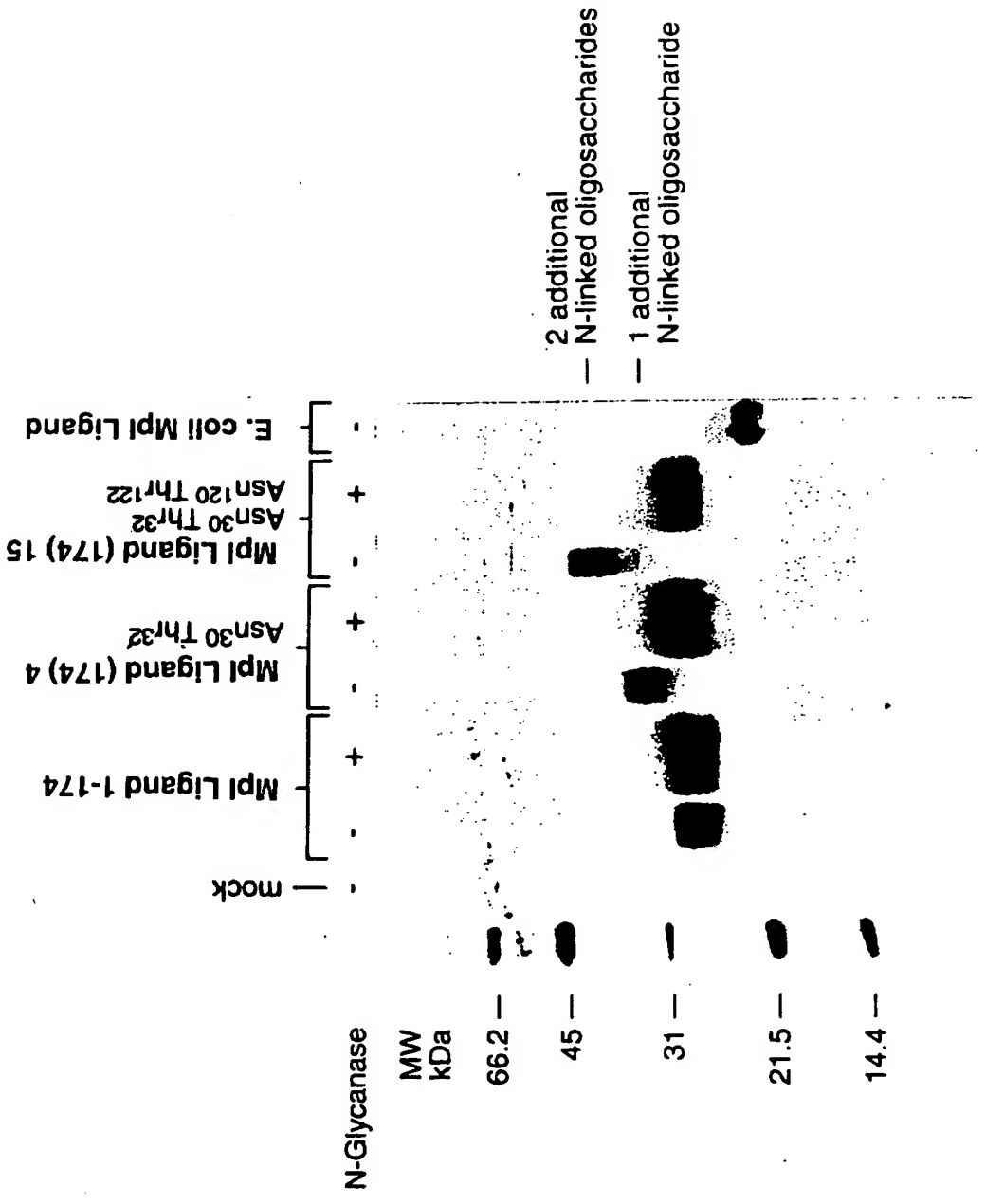
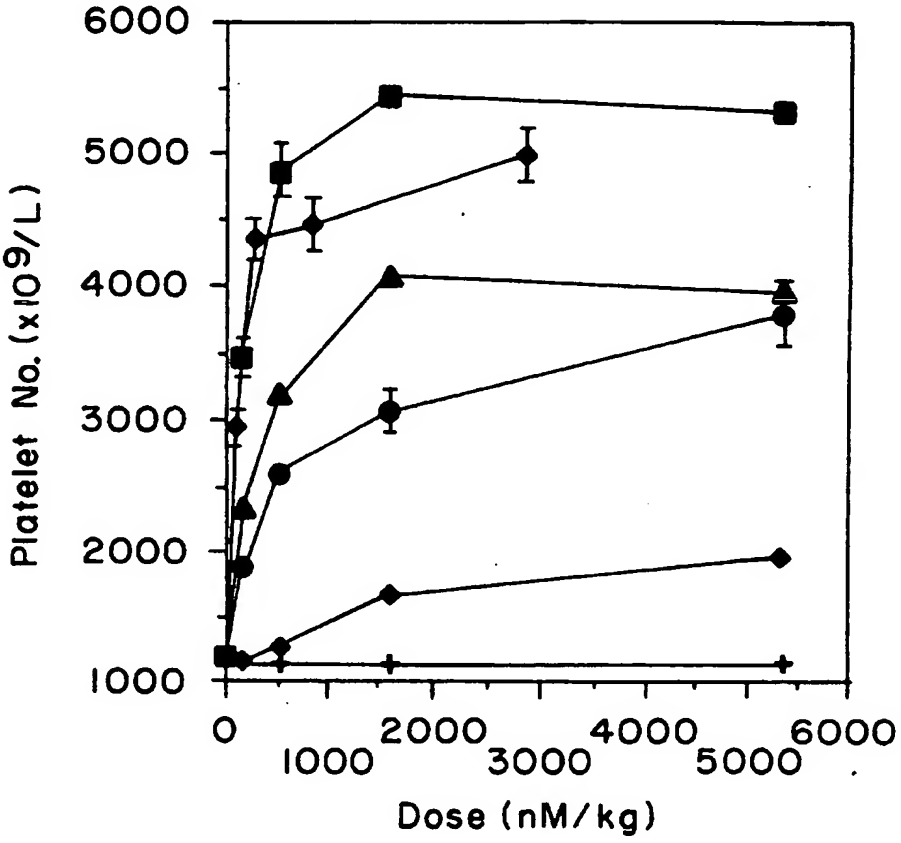

2 additional N-linked oligosaccharides
1 additional N-linked oligosaccharide
E. coli Mpl Ligand
Mpl Ligand (174) 15 Asn30 Thr32 Asn120 Thr122
Mpl Ligand (174) 4 Asn30 Thr32
Mpl Ligand 1-174
mock
N-Glycanase
MW kDa
66.2
45
31
21.5
14.4

FIG.10

FIG. 11


6000
5000
4000
3000
2000
1000
Platelet No. (x10⁹/L)
0
1000
2000
3000
4000
5000
6000
Dose (nM/kg)
5. CHO-174-15
2. CHO-1-332
4. CHO-174-4
3. CHO 1-174
6. E coli Mk 1-174
1. Carrier

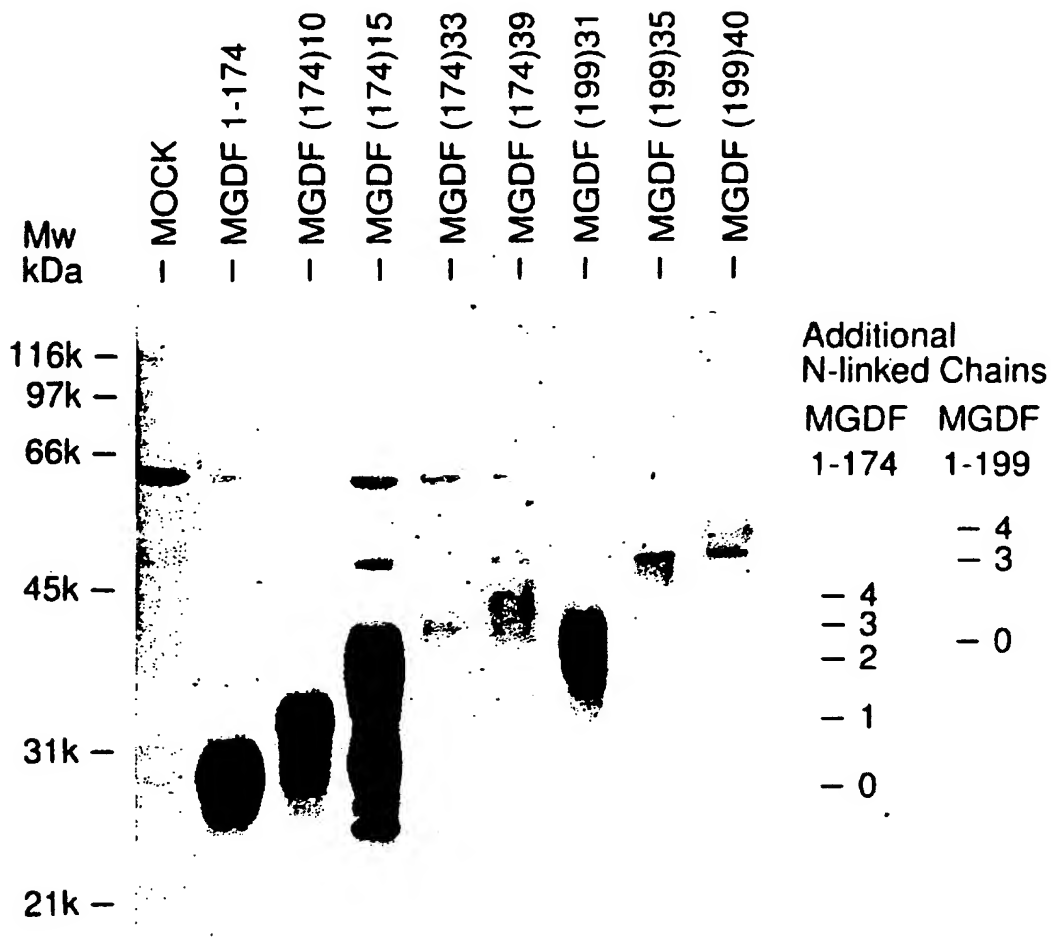

Mw
kDa
– MOCK
– MGDF 1-174
– MGDF (174)10
– MGDF (174)15
– MGDF (174)33
– MGDF (174)39
– MGDF (199)31
– MGDF (199)35
– MGDF (199)40
116k –
97k –
66k –
45k –
31k –
21k –
Additional
N-linked Chains
MGDF 1-174
– 4
– 3
– 2
– 1
– 0
MGDF 1-199
– 4
– 3
– 0

FIG.12

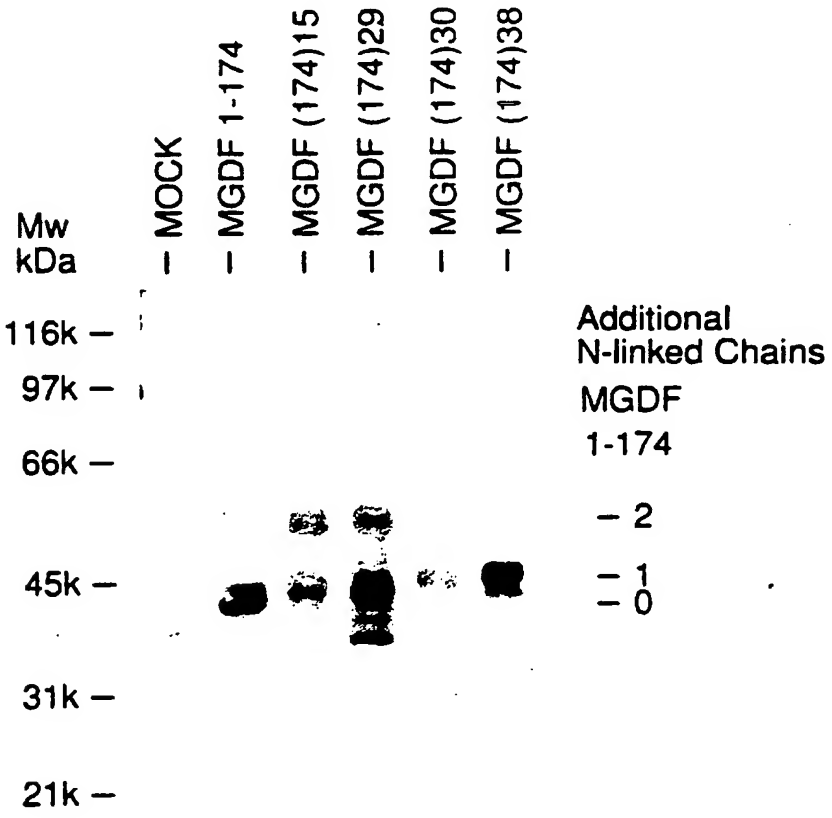

Mw
kDa
MOCK
MGDF 1-174
MGDF (174)15
MGDF (174)29
MGDF (174)30
MGDF (174)38
116k
97k
66k
45k
31k
21k
Additional
N-linked Chains
MGDF
1-174
2
1
0

FIG.13

Brown F, Mire-Sluis AR (eds): Biological Characterization and Assay of Cytokines and Growth Factors. Dev Biol Stand. Basel, Karger, 1999, vol 97, pp 39-47

# Chromatography of Recombinant Proteins

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**Abstract:** Variants of intact polypeptides/proteins ranging in mass from 6,500 to 70,000 Da were easily separated using reversed-phaseHPLC (rpHPLC) or affinity chromatography. A variant of rhIGF-I, where the racemization of a serine residue was detected in the intact molecule, was resolved from rhIGF-I within 25 minutes by rpHPLC. Other variants of rhIGF-I separated by this method include methionine sulphoxide at position 59, des $Gly^1$, des $Gly^1Pro^2$, Glu for Asp substitution at position 20 and incorrectly folded IGF-I. For rhDNase (~ 40 kDa), affinity chromatography was able to clearly resolve three different amino acids (Asn, Asp and iso-Asp) at position 74 of the intact glycoprotein. The presence or absence of O-linked sugars on Thr -37 of recombinant human thrombopoietin was rapidly demonstrated by rpHPLC.
While the separation of these types of variants is essential, the demonstration of biological activity is critical for designing specifications that allow the administration of these proteins into humans. Once a correlation exists between the variant and its biological activity, control of the manufacturing process can be better achieved with analytical methodology.

## Introduction

The advent of recombinant DNA technology has resulted in the initiation of multiple clinical trials in the areas of oncology, endocrinology, and cardiovascular disease. In the last 10 years, over a dozen therapeutic recombinant proteins have been licensed and hundreds more have entered clinical trials.

One of the most critical parameters to control during production is the introduction of variants of the desired protein into the manufacturing process. Proteins are subject to a variety of post-translational modifications (e.g. glycosylation, phosphorylation, oxidation, deamidation, proteolytic cleavage, gamma carboxylation). In addition, point mutations [1] and unexpected substitutions (e.g. norleucine for methionine) arising from fermentation conditions have also been described for recombinant proteins [2].

The separation and corresponding biological activity of variants of three proteins recombinant human insulin-like growth factor-I (rhIGF-I), recombinant human deoxyribonuclease I (rhDNase I), and recombinant human thrombopoietin (rhTPO) will be shown. Finally, the role of analytical methodology in the design of a strong control system for the manufacture of recombinant protein therapeutics will be discussed.



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